DNA Analysis Unit II
Mitochondrial DNA Analysis Protocol

1 Scope

Mitochondrial DNA (mtDNA) analysis will be performed on a variety of biological tissues. State and local contributors should call the DNAU II Unit Chief or an Examiner prior to submitting a case requiring mtDNA analysis to obtain approval for submission of evidence. The submitting agency must provide known blood, saliva and/or hair standards, as appropriate. Analysis of cases submitted without known samples will proceed only at the discretion of the Unit Chief. The known samples should be packaged separately from the questioned samples.

1.1 Hairs

Hairs will be sent to the Trace Evidence Unit (TEU) prior to being subjected to mtDNA analysis. Hairs associated through comparison microscopy in TEU will be analyzed. Other human hairs may be subjected to mtDNA analysis as deemed necessary. Hairs that have been microscopically compared prior to their submission to the FBI Laboratory will be examined by TEU.

1.2 Bones and Teeth

Refer to the Procedure for DNA Extraction from Calcified Tissue (Section 3.3) for details on submission of bones and teeth.

1.3 Bodily Fluids and Tissue Samples

Bodily fluids and tissue samples will be subjected to mtDNA analysis at the discretion of the Unit Chief or a DNAU II Examiner.

2 Quality Assurance

The Unit's Quality Assurance Manual describes in detail the Quality Assurance program of the Unit. The program's objective is to continuously monitor, and improve where necessary, the quality of mtDNA analysis. Described below are Unit protocol-related procedures critical to ensuring a quality work product. The sensitivity of mtDNA analysis makes it especially important that particular procedures are adhered to in the Laboratory to minimize the potential for laboratory-induced DNA contamination. That, along with the use and interpretation of control samples, quality controlled reagents, the calibration and maintenance of instruments and equipment, and the review of cases, comprise the essential elements of protocol-related quality

assurance procedures.

Problems arising related to materials, supplies, and equipment are addressed in a timely manner, regardless of the schedule of the prescribed checks, calibrations, or maintenance described in this section.

2.1 Evidence Handling, Extraction, Amplification, and Post-Amplification Practices

1. Extraction and amplification set-up areas are separated from post-amplification work areas. If work is performed by an individual in the post-amplification work areas during a particular day, no extractions or amplification set-ups should be done later in that day by that individual. Amplified mtDNA must not be moved from the post-amplification work areas into the extraction/amplification set-up areas. Laboratory coats used in the post-amplification work areas are not moved to the extraction and amplification set-up areas prior to laundering.

2. Dedicated laboratory coats, disposable gloves, clean room face masks, and protective eye wear when appropriate, are worn at all times during extraction and amplification set-up procedures. Dedicated laboratory coats, disposable gloves, safety glasses, and face masks when appropriate, are worn at all times during the post-amplification procedures.

3. Biologists should have and use their own sets of pre-amplification and post-amplification pipettes.

4. Pipettes, aerosol resistant pipette tips, scalpels, scissors, forceps, extraction, and amplification reagents are dedicated for use in mtDNA extraction/amplification set-up.

5. All glassware and suitable plastic containers are autoclaved. Refer to the user's manual for details on the use and maintenance of the autoclave.

6. Only one case and only one item of evidence from that case should be opened at a time. Items of questioned origin typically are extracted and amplified prior to items of known origin.

7. All extraction and amplification set-up procedures are performed in an AirClean hood or a dedicated laminar flow hood with an ultraviolet (UV) light source. Bones and teeth are abraded and cut under dedicated hoods.

8. Work surfaces in the extraction/amplification set-up area are thoroughly cleaned with a 10% household bleach solution (made fresh each day of use) before and after processing an item of evidence.

9. Work spaces under the dedicated hoods in the extraction and amplification set-up areas are exposed to UV light for a minimum of 15 minutes before processing a sample.

10. All appropriate reagents and all supplies used for extraction and amplification are exposed to UV light in the laminar flow hood, an AirClean hood, or in a UV crosslinker for a minimum of 15 minutes prior to use. Tubes for cycle sequencing, and water used for post-amplification procedures are subjected to UV crosslinking treatment as well.

11. Pipetters used for extraction and amplification set-up are wiped with alcohol swabs or 10% bleach and exposed to UV light for a minimum of 15 minutes before use. Pipetters used for post-amplification procedures are wiped with alcohol swabs before use. Tube racks returned to the pre-amplification work space after being stored in the post-amplification work space are wiped with 10% bleach and exposed to UV light for a minimum of 15 minutes before re-use in the pre-amplification work space.

12. Scissors and forceps are cleaned with alcohol swabs and exposed to UV light for a minimum of 15 minutes before use. Scalpel blades are used once and discarded into a sharps container.

13. Pipette tips are changed between each reagent and sample addition.

14. To reduce the potential for aerosols, the last bit of sample from a pipette should not be "blown out".

15. All amplification reagent tubes should be centrifuged briefly before opening.

16. DNA is the final component added to the amplification reaction mix.

17. Reagents are stored as small aliquots to minimize the number of times a given reagent tube is opened.

18. A reagent blank (RB) is processed with each evidentiary sample. A negative control (NC) and a positive control are set up in parallel with each sample and RB amplification.

19. The lot numbers of appropriate reagents used in each set of samples are recorded in the case notes.

20. The pH meters are checked each day of use by performing a two-point pH standardization with pH buffers (typically pH 7 and 10) according to the manufacturer's instructions. The checks are documented in the pH meter log books.

2.2 Quality Control of Reagents

The following identifies the critical reagents used in the mtDNA analysis and describes quality control procedures to ensure suitability for casework. Reagents are labeled with the date received or the date prepared, the initials of the preparer (where appropriate), the reagent name, the lot number, the QC date, the initials of the person performing the QC, the date the reagent is first used in the laboratory (start date) and the initials of the person first using it, the expiration date (1 year from QC date if not otherwise specified), and the storage conditions. All critical reagents are tested from the appropriate point through sequencing. For extraction and amplification critical reagents, if multiple reagent blank and negative control samples are run, the majority of the reagent blank and negative control samples must be "not analyzable" after sequencing. Any reagent blank or negative control samples containing analyzable sequence must be different from the sample being tested by at least one base. QC sequences must be analyzed by the Quality Assurance Program Manager or a designated caseworking examiner and must yield high-quality electropherograms and the correct sequence. The Examiner initials the sequence sample sheet, the project file sheet, and the sequence difference sheet, indicating in writing that the reagent is acceptable for use in casework. The Quality Assurance Biologist will be notified by the Quality Assurance Program Manager if any reagent fails QC or demonstrates possible problems after passing QC. The Quality Assurance Program Manager reviews the reagent QC documentation and directs and documents any action deemed necessary as a result of reagent QC checks.

2.2.1 Extraction Critical Reagents

1. Stain Extraction Buffer (SEB)

2. Proteinase K

3. DTT (Dithiothreitol)

4. PCIA (Phenol/Chloroform/Isoamyl Alcohol 25:24:1 v/v)

5. AL, AW1, and AW2 buffers in the QIAamp DNA Mini Kits

The above reagents are checked by extracting a known blood/bloodstain sample (typically a training or proficiency sample). The sample is amplified at HV1 or HV2, quantified by capillary electrophoresis and sequenced. SEB is typically aliquoted into 15 mL tubes. Each tube contains approximately 5 mL and is stored at room temperature. Proteinase K is typically aliquoted into 500 µL tubes. Each tube contains approximately 5 uL and is stored at 4 ^{o}C. DTT and PCIA are stored at 4 ^{o}C. The buffers from the QIAamp DNA Mini kits are stored at room temperature. The container housing each reagent is labeled as indicated in Section 2.2. Sequences must be analyzed by the Quality Assurance Program Manager or a designated caseworking Examiner. Records of the check are maintained. The

procedure summary sheet indicates that the lot is acceptable for use in casework.

2.2.2 Amplification Critical Reagents

1. 10X PCR Buffer, AmpliTaq Gold, dNTPs, Bovine Serum Albumin (BSA)

   Amplification reagents are tested by amplifying HV1 or HV2 of a positive control sample, quantifying by capillary electrophoresis and sequencing. Tubes of 10X PCR Buffer, AmpliTaq Gold, and dNTPs should be stored at -20°C. BSA is prepared as indicated in Section 10.1. Aliquots should be labeled and stored at -20°C. The container housing each reagent is labeled as indicated in Section 2.2. Sequences must be analyzed by the Quality Assurance Program Manager or a designated caseworking Examiner. Records of the check are maintained. The procedure summary sheet indicates that the lot is acceptable for use in casework.

2. Positive Control

   New lots and dilutions of HL60 positive control DNA are tested by amplification of HV1 and HV2, quantification by capillary electrophoresis, and sequencing. The HL60 DNA is diluted to a final concentration of 10 pg/µL. This dilution is typically aliquoted into 500 µL tubes. Each tube typically contains approximately 45 µL. Aliquots should be labeled as indicated in Section 2.2 and stored at -20°C or colder. The container housing the positive control DNA aliquots is labeled as indicated in Section 2.2. Sequences must be analyzed by the Quality Assurance Program Manager or a designated caseworking Examiner. Records of the check are maintained. The procedure summary sheet indicates that the lot is acceptable for use in casework.

3. Amplification Primers

   New lots of amplification primers are tested by amplifying the positive control DNA with the appropriate primer pair, quantification by capillary electrophoresis, and sequencing. Amplification primers are initially hydrated with sterile deionized water to a stock concentration of 100 µM. The stock concentration is diluted to a working concentration of 30 µM (A1, A2, B1, B2, C1, C2, D1, D2, 617) or 10 µM (miniprimers) as appropriate. Amplification primers are typically aliquoted into 500 µL tubes. Each tube contains approximately 10 µL. Aliquots should be stored at -20°C and the containers housing the tubes labeled as indicated in Section 2.2. Sequences must be analyzed by the Quality Assurance Program Manager or a designated caseworking Examiner. Records of the check are maintained. The procedure summary sheet indicates that the lot is acceptable for use in casework.

4. EXOSap-IT

EXOSap-IT is tested by amplifying one region of a positive control, subjecting it to the EXOSap-IT procedure, quantifying by capillary electrophoresis, and sequencing. Tubes of EXOSap-IT should be stored at -20°C and the containers housing the tubes labeled as indicated in Section 2.2. Sequences must be analyzed by the Quality Assurance Program Manager or a designated caseworking Examiner. Records of the check are maintained. The procedure summary sheet indicates that the lot is acceptable for use in casework.

2.2.3 Sequencing Critical Reagents

1. Sequencing Kits

New lots of sequencing kits are tested by sequencing either HV1 or HV2 of the positive control DNA. Sequencing kits should be stored at -20°C and the containers housing the tubes labeled as indicated in Section 2.2. Sequences must be analyzed by the Quality Assurance Program Manager or a designated caseworking Examiner. Records of the check are maintained. The procedure summary sheet indicates that the lot is acceptable for use in casework.

2. 1 μM Primers

New lots of primers used in cycle sequencing are diluted from the 100 μM primer stocks. A cycle sequencing reaction using the appropriate region of the positive control DNA is performed using each diluted primer. Typically, 16 μL aliquots of the 1 μM primer stock are placed into 500 μL tubes. Aliquots are stored at -20°C and the containers housing the tubes labeled as indicated in Section 2.2. Sequences must be analyzed by the Quality Assurance Program Manager or a designated caseworking Examiner. Records of the check are maintained. The procedure summary sheet indicates that the lot is acceptable for use in casework.

3. Formamide

New lots of formamide are tested by sequencing either HV1 or HV2 of the positive control DNA. Aliquots are stored at -20°C and the containers housing the tubes labeled as indicated in Section 2.2. Sequences must be analyzed by the Quality Assurance Program Manager or a designated caseworking Examiner. Records of the check are maintained. The procedure summary sheet indicates that the lot is acceptable for use in casework.

2.3 Instrument/Equipment Calibration and Maintenance

The appropriate Instrumentation Biologist will be notified if any instrument fails QC or

demonstrates possible problems after passing QC. The Quality Assurance Program Manager reviews the calibration and maintenance/service documentation and directs and documents any action deemed necessary as a result of calibration checks, maintenance, or service.

2.3.1 Thermal Cycler

1. The sample block is cleaned quarterly. Refer to "Cleaning the sample wells" and "Cleaning the sample block cover" procedures described in the Maintenance section of the Users Manual for the GeneAmp PCR System 9700. Records of the cleaning are maintained.

2. The specified tests described in the Maintenance section of the Users Manual for the GeneAmp PCR System 9700 are run quarterly using the 0.2 mL Temperature Verification Kit (Applied Biosystems). This system is sent to an appropriate vendor annually for recalibration to a National Institute of Standards and Technology (NIST) standard. Recalibration records are maintained.

3. The GeneAmp PCR System 9700 is subjected to repairs as needed

2.3.2 Incubators

1. The incubators used in the calcified tissue extraction procedure, as well as the Vortemps or Thermomixers used for the extraction procedure, are checked quarterly using thermometers described in 2.3.8. The temperature is recorded, and the record is dated and initialed by the operator on a temperature log sheet. These records are maintained.

2.3.3 Hoods

1. Maintenance by an outside vendor is performed annually on the biosafety cabinets and other hoods with air flow (except hoods used for grinding bones and teeth). This includes filter changes where necessary. Records are maintained.

2. The UV lights in the biosafety cabinets, AirClean, and dead space hoods are changed yearly.

3. The prefilters in the hoods used for DNA extraction, amplification, and phenol/chloroform/isoamyl alcohol (PCIA) extraction are changed semiannually. The replacement checklist will be initialed and dated by the operator at the time of replacement. The prefilters in the calcified tissue extraction hoods are changed after each use. The HEPA filters in the calcified tissue extraction hoods are changed as necessary. The HEPA filters in the AirClean hoods used for DNA extraction and amplification, as well as the carbon filter in the AirClean hood used for PCIA, are certified annually by a hood

technician and changed as necessary. Records are maintained.

2.3.4 UV Crosslinker

1. The UV lights in the Spectrolinker UV crosslinkers are checked quarterly by running the Autocrosslink function (press "autocrosslink" then "start") and ensuring the cycle completes in a minute or less. Additionally, the UV lights are observed through the window in the door to determine they are all on. **CAUTION: Wear UV-protective eyewear when observing the UV lights.** The UV lights in the Spectrolinker UV crosslinkers are checked quarterly by running the intensity check (press "intensity" then "start"). A reading of 1500 or greater indicates the bulbs are functioning properly. UV lights will be replaced when indicated by the results of the check. Records are maintained.

2.3.5 Agilent Bioanalyzer Capillary Electrophoresis (CE) Instrument for Post-Amplification Quantification

1. Quarterly maintenance is performed on the Agilent Bioanalyzers as indicated in the manufacturer's instructions, to include cleaning and/or changing of the 16-pin Electrode cartridge, cleaning of the lens, and replacing the syringe and syringe adapter of the chip priming station. Records are maintained.

2.3.6 Refrigerators/Freezers

1. The temperature of refrigerators and freezers are checked daily (on work days) using thermometers described in 2.3.8 and the results recorded. If a daily check is missed, it is noted on the log sheet. An out-of-range temperature will be circled, and the temperature checked again at least one more time during that day to determine whether it has come back into range. Acceptable ranges for refrigerators set to 4^{o}C are 0-10^{o}C, -30 to -5^{o}C for freezers set to -20^{o}C, and -90 to -70^{o}C for freezers set to -80^{o}C. These ranges are set to accommodate normal fluctuations of refrigerators and freezers due to their cycling and use, but it is expected that refrigerators and freezers will be near or at the set point during longer periods of non-use. A determination of whether a refrigerator or freezer needs repair is based on a pattern of temperature readings falling outside of, or near the limits of, the acceptable ranges, and is made by the Instrumentation Biologists.

2. The NIST-traceable thermometers used to monitor unit refrigerators and freezers are replaced as necessary when their calibration expires or the calibration may be checked against a certified purchased thermometer. This is done by placing the certified thermometer and the thermometer being checked in the freezer or refrigerator, leaving them in for at least one hour, and recording the temperature on both thermometers. An acceptable difference between readings from the two thermometers is ± 2^{o}C. If the refrigerator or freezer thermometer is out of this range, continue to monitor for another day.

Replace refrigerator or freezer thermometer if necessary. Records are maintained.

3. Maintenance is performed by outside vendors on refrigerators and freezers when necessary. Records are maintained.

2.3.7 Pipetters

1. Pipetters are subjected to annual calibration and maintenance by an outside vendor. Records are maintained.

2.3.8 Thermometers

1. Thermometers used for incubator, refrigerator, and freezer checks are NIST traceable, and are certified as such from the supplier. Certified thermometers may be replaced as necessary or may be checked in the laboratory against a certified purchased thermometer as detailed in Section 2.3.6. Records are maintained.

2.3.9 ABI Prism 3130 Genetic Analyzers

1. New capillary arrays are tested by performing sequencing runs using one primer to sequence HL60 HV1 or HV2. High quality sequence data must be obtained, and, for the arrays, consistent results must be obtained from the 16 capillaries. Sequences must be analyzed by the Quality Assurance Program Manager or a designated caseworking Examiner. Records of the check are maintained.

2. Spatial calibrations are performed when a capillary is installed or replaced. They should also be performed when the array window has been temporarily removed from the detection block. Refer to Chapter 2 of the ABI Prism 3130 Genetic Analyzers Getting Started Guide for instructions on performing and evaluating spatial calibrations.

3. Spectral calibrations are performed when the instrument's laser or charged coupled device (CCD) camera has been adjusted by an ABI service engineer and when a new dye set is used on an instrument. They are also performed following the yearly preventative maintenance completed by an ABI service engineer if he/she adjusts the laser or CCD camera, and are recommended when a decrease in spectral separation is seen in sequence analysis (typically seen as pull-up background peaks). Refer to Chapter 3 of the ABI Prism 3130 Genetic Analyzers Getting Started Guide for instructions on performing spectral calibrations. Following spectral calibration, the instruments are tested by performing sequencing runs using one primer to sequence HL60 HV1 or HV2. High quality sequence data must be obtained, and, for the arrays, consistent results must be obtained from the 16 capillaries. Sequences must be analyzed by the Quality Assurance Program Manager or a designated caseworking Examiner. Records of the check are maintained.

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4. Yearly preventative maintenance by Applied Biosystems is performed on the ABI Prism 3130 Genetic Analyzer instruments. Records are maintained.

2.3.10 Microcentrifuges

1. No routine calibration or maintenance is required for microcentrifuges. G-forces referenced in this protocol are intended to be approximate and exact determination of g-force in a particular microcentrifuge is not necessary. Refer to the manufacturer's information for particular microcentrifuges if it is necessary to convert rpm numbers to g-forces.

2.4 Additional Quality Assurance Measures

2.4.1 Cycle Sequencing

1. The positive control run with each evidentiary sample serves as a check of the cycle sequencing reaction.

2.4.2 Sequence analysis

1. Both light (L) and heavy (H) strand sequencing reactions are performed to provide confirmatory data.

2. In cases where there is sequence concordance between samples of questioned and known origin, for any samples which will be entered into the Combined DNA Index System (CODIS), and for all TEDAC samples, the sequences are analyzed independently by at least two qualified Examiners (refer to Section 8.3.4). The completion of these confirmations is recorded in the case notes.

2.4.3 Technical Review

1. All reports for cases for which laboratory procedures have been performed are technically reviewed by another qualified Examiner prior to their issuance. Technical review is documented on the Technical Review checklist, which is maintained with the case notes.

2.4.4 Administrative Review

1. All reports are administratively reviewed by the Unit Chief or his/her designee prior to their issuance. Administrative review is documented on the Administrative Review checklist, which is maintained with the case notes.

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2.4.5 NIST Standard Reference Material

1. The extracted human DNA component of the NIST Standard Reference Material 2392-I (HL60) is amplified and sequenced for the whole control region (WCR) of the mitochondrial DNA. The HL60 positive control used for casework is amplified and sequenced in parallel with the NIST SRM 2392-I. This check is performed yearly.

2.5 Interpretation of Control Samples

2.5.1 Reagent Blanks

1. The reagent blank (RB) monitors for the presence of exogenous DNA from the extraction steps through sequencing and is processed from the DNA extraction procedure through sequencing procedure along with the evidentiary sample.

2. The mtDNA analysis of a particular sample extract does not proceed if the amount of amplified mtDNA in the RB exceeds 10% of the amount of amplified mtDNA in the sample as assessed through capillary electrophoretic (CE) quantification.

3. Samples may be re-extracted with new reagents should the amount of amplified mtDNA in the RB exceed 10% of the amount of amplified mtDNA in the sample or have sequence concordance with the sample. A new RB is included in the repeated extraction.

4. Regardless of the relative quantity of mtDNA in the RB and the sample, if the RB sequence is in concordance with the sample, the results of the sample amplification reaction are not used for comparison purposes. The evidence sample sequence can be used under the following conditions:

    a) the RB does not analyze;
    b) the RB analyzes but is not of requisite quality for comparison purposes;
    c) the RB sequence analyzes, but the sequence is not in concordance with the evidence sample and its quantity is less than or equal to 10% of that of the evidence sample.

5. If analyzable mtDNA sequence is obtained from the reagent blank, an attempt is made to determine the source of this DNA.

2.5.2 Negative Control

1. The negative control (NC) monitors for the presence of exogenous DNA in the amplification reagents, from the amplification procedure through the sequencing procedure.

2. The mtDNA analysis of a particular sample extract does not proceed if the amount of amplified mtDNA in the NC exceeds 10% of the amount of amplified mtDNA in the sample as assessed through CE quantification.

3. Samples may be re-amplified with new reagents should the amount of amplified mtDNA in the NC exceed 10% of the amount of amplified mtDNA in the sample or have sequence concordance with the sample. A new NC is included in the repeated amplification.

4. Regardless of the relative quantity of mtDNA in the NC, if the NC sequence is in concordance with the evidence sample, the results of the sample amplification reaction are not used for comparison purposes. For example, if the NC for region HV1A shows a sequencing result which is in concordance with an HV1A sample sequence, all HV1A sequences are not used for comparison purposes. The evidence sample sequence can be used under the following conditions:

   a) the NC does not analyze;
   b) the NC analyzes but is not of requisite quality for comparison purposes;
   c) the NC sequence analyzes, but the sequence is not in concordance with the evidence
   sample and its quantity is less than or equal to 10% of that of the evidence sample.

5. If analyzable mtDNA sequence is obtained from the negative control, an attempt is made to determine the source of this DNA.

2.5.3 Positive Control

1. The use of a positive control (see Appendix A) monitors the success of amplification, sequencing, and sequence analysis.

2. The record of the particular positive control used and its lot number is maintained for each case.

3. The mtDNA analysis of a particular sample extract is repeated, going back to the appropriate step, if the positive control fails to amplify and/or sequence correctly.

3 Procedures for DNA Extraction

This section describes DNA extraction procedures from hair, body fluids and tissues, and calcified tissues (bones and teeth). Whenever possible, a portion of the sample will be retained. All extraction procedures are performed in dedicated laminar flow or AirClean hoods.

3.1 Procedure for DNA Extraction from Hair

3.1.1 Cleaning and Preparation of Micro Tissue Grinders

Micro Tissue Grinders are used to grind hairs for DNA extraction. The grinders consist of matched sets of mortars and pestles, and are used as such. Grinders are used one time and discarded. To facilitate working with the grinders, the grinders may be placed in a small plastic tube, such as a 1.5 mL Sarstedt tube. Prior to beginning hair extraction procedures, the grinders are cleaned as follows:

1. Carefully rinse the grinder with distilled, deionized water (ddH_2O). Using cotton tip applicators and 5% (w/v) Terg-a-zyme detergent (0.5 g Terg-a-zyme in 10 mL water; it may be warmed) scrub the pestle and the insides of the mortar. Repeat the application of Terg-a-zyme with the swab two additional times.

2. Rinse the grinder with ddH_2O and add approximately 300 µL of 4 N sulfuric acid (H_2SO_4). **CAUTION: Sulfuric acid is caustic. Gloves, protective eye wear, and a laboratory coat must be worn, and the addition to the grinder is performed under a fume hood.** Place the pestle in the matching mortar and briefly simulate grinding. Allow the mortar and pestle to soak in 4 N H_2SO_4 under the fume hood for a minimum of 20 minutes.

3. Rinse the grinder with ddH_2O. Remove the pestle and place the mortar in a microcentrifuge. Pulse spin at high speed to collect the remaining water. Remove the remaining water from the mortar with a pipette.

4. Place the grinder in a rack and place in a UV crosslinker, subjecting it to UV light for a minimum of 15 minutes. Arrange them within the crosslinker in such a way as to expose them to the UV light.

3.1.2 DNA Extraction

1. Prepare the reagent blank by placing 200 µL of prepared stain extraction buffer (SEB) (Section 10.1) into the grinder. Briefly simulate grinding. Remove the pestle and transfer the liquid to a sterile 1.5 mL plastic tube. Add 1 µL of 600 U/mL of proteinase K. Vortex briefly. Place tube in designated Vortemp or Thermomixer and incubate at 56°C for 2 hours to overnight.

2. Examine the hair to be extracted under a stereomicroscope for the possible presence of sheath material, surface debris, or bodily fluids. If sheath material is present, the root end may be removed and sent for nuclear DNA analysis at the Examiner's discretion.

3. Depending on the nature of the evidence and at the Examiner's discretion, hairs that were previously mounted on a microscope slide, and/or have water and ethanol insoluble material adhering to them may be treated as follows. Remove the hair from the tube and place the hair into an extraction tube containing enough xylene (or a xylene substitute) to cover the hair. Place the tube in an ultrasonic water bath for approximately 20 minutes.

4. Prepare a wash solution of 5% (w/v) Terg-a-zyme detergent. Place the tube of solution in a UV crosslinker and subject it to UV light for a minimum of 15 minutes.

5. Place a portion of the hair (approximately 2 cm and from the root end where possible; a smaller or larger portion may be used at the Examiner's and Biologist's discretion where appropriate) in a 1.5 mL plastic tube filled with approximately 1 mL of 5% Terg-a-zyme and place in a rack in an ultrasonic water bath for at least 20 minutes. Additional washes using fresh aliquots of the Terg-a-zyme solution may be performed at the discretion of the Examiner and Biologist based on the nature of the evidence. The hair may be examined again under the stereomicroscope for the presence of surface debris.

6. Briefly rinse the hair in approximately 1 mL 100% ethanol, followed by 1 mL ddH_2O, both of which have been placed in a UV crosslinker and exposed to UV for a minimum of 15 minutes prior to adding the hair.

7. To the same grinder add 200 µL of SEB. Place the hair fragment into the grinder.

8. Move the pestle up and down to force the hair into the bottom of the mortar. Grind until fragments are no longer visible.

9. Remove the pestle from the mortar. If liquid is adhering to the pestle head, it may be gently passed along the inner lip of the mortar until liquid flows to the bottom of the mortar.

10. Transfer the homogenate liquid to a sterile 1.5 mL plastic tube. Discard the grinder in the glass disposal box.

11. Add 1 µL of 600 U/mL of proteinase K to the sample.

12. Vortex briefly. Place the tube in a designated Vortemp or Thermomixer and incubate at 56^{o}C for 2 hours to overnight.

13. Remove the tubes from the Vortemp or Thermomixer and briefly spin in a microcentrifuge to force the condensate into the bottom of the tubes.

14. Add 200 µL of phenol/chloroform/isoamyl alcohol (PCIA, 25:24:1) to the sample and reagent blank tubes.

CAUTION: PCIA is an irritant and is toxic. Its use must be confined to the designated hood. Gloves and a mask must be worn.

15. Vortex briefly to attain a milky emulsion, then spin the tubes in a microcentrifuge for 3 minutes at a setting of 10000 X g.

16. Assemble and label a Microcon 100 unit for each sample (see Microcon Operating Instructions). Place unit in a UV crosslinker and expose to UV light for a minimum of 15 minutes prior to use. Add 200 µL of ddH_2O to the filter side (top) of each Microcon 100 concentrator.

17. Carefully remove the aqueous phase (approximately 200 µL) from each tube, and transfer to the appropriate concentrator. (Avoid drawing any of the proteinaceous interface into the pipette tip.)

18. Spin the Microcon 100 concentrators for 5 minutes at a setting of 3000 X g. (Note: additional spin time and/or higher speed, up to 4500 X g, may be required to filter the entire volume.)

19. Discard the wash and return the filtrate cups to the concentrators.

20. Add 400 µL of filtered ddH_2O to the filter side of each Microcon 100 concentrator.

21. Spin again at a setting of 3000 X g for 5 minutes and discard the filtrate cups. (Note: additional spin time and/or higher speed, up to 4500 X g, may be required to filter the entire volume.)

22. Add 60 µL of hot (80^o-90^oC) ddH_2O to the filter side of each Microcon 100 concentrator and place a retentate cup on the top of each concentrator.

23. Briefly vortex each Microcon 100 concentrator with the retentate cup pointing upward.

24. Invert each concentrator with its retentate cup and spin in a microcentrifuge at a setting of 10000 X g for 3 minutes.

25. Discard the concentrators. Using a pipetter, measure the volume of the retentate. Add sterile water if necessary to bring volume to 60 µL. Cap the retentate cups.

26. The samples can then be amplified. Store extracted DNA at 4^oC.

3.2 Procedure for DNA Extraction from Body Fluids and Tissues

Body fluid samples can be made into swabbings, or stains on cotton sheeting, FTA paper, or filter paper (see Appendix B for procedure for preparing bloodstains from liquid bloods). Alternatively, liquid blood may be extracted directly. Stains or swabbings are air dried before use. Dried stains and liquid blood should be stored at 4°C. Liquid blood tubes should be stored packaged in mailing tubes designed for that purpose. Tissue samples may be rinsed briefly in 1% sodium dodecyl sulfate (SDS) or a xylene substitute, such as Xyless, prior to extraction at the discretion of the Examiner and Biologist based on the nature of the evidence. This is done by placing the tissue in a 15 mL capped, sterile, disposable tube and inverting several times. At the Examiner's and Biologist's discretion, as an alternative to PCIA extraction and Microcon 100 filtration (Steps 8 -17 in this section), the DNA extract may be purified using the QIAamp procedure described in Section 3.3.7. Refer to step 18 of this section for guidance on dilutions of extracts for amplification.

1. Prepare a reagent blank for each extraction procedure by adding 300 µL of prepared SEB (Section 10.1) and 2 µL of 600 U/mL of proteinase K to a 1.5 mL microcentrifuge tube. Briefly vortex and place tube in designated Vortemp or Thermomixer and incubate at 56°C for 2 hours to overnight.

2. Add 300 µL of SEB to a 1.5 mL microcentrifuge tube. Add sample to tube as follows. For stains or tissue samples, use a small portion (approx. 3 mm x 3 mm). For buccal swabs, use approximately one half of the swab. For liquid blood, use 5 µL.

3. Add 2 µL of 600 U/mL of proteinase K. Briefly vortex and spin in a microcentrifuge to force the sample into the liquid.

4. Incubate the tube at 56°C for 2 hours to overnight. Briefly spin the tubes in a microcentrifuge to force condensate into the bottom of the tubes.

5. Transfer the cutting, swab or remaining undigested tissue into an extraction tube filter basket insert that has been exposed to UV light in a crosslinker for a minimum of 15 minutes prior to use. Place the filter insert into the same tube containing the stain extract. Briefly spin at high speed. Remove and discard the cutting and the filter (use appropriate biohazard disposal container where necessary).

6. Add 300 µL PCIA to the reagent blank and sample tubes.

   CAUTION: PCIA is an irritant and is toxic. Its use must be confined to the designated fume hood. Gloves and a mask must be worn.

7. Vortex the tubes to attain a milky emulsion, then spin in a microcentrifuge for 3 minutes at

a setting of 10000 X g.

8. Assemble and label a Microcon 100 unit for each sample (see Microcon Operating Instructions). Place unit in a UV crosslinker and expose to UV light for a minimum of 15 minutes prior to use. Add 100 µL of ddH_2O to the filter side (top) of each Microcon 100 concentrator.

9. Carefully remove the aqueous phase (approximately 300 µL) from each tube, and transfer to the appropriate concentrator. (Avoid drawing any of the proteinaceous interface into the pipette tip.) PCIA extraction may be repeated if necessary, until the interface is clean.

10. Spin the Microcon 100 concentrators for 5 minutes at a setting of 3000 X g (Note: additional spin time and/or higher speed, up to 4500 X g, may be required to filter the entire volume).

11. Discard the wash and return the filtrate cups to the concentrators.

12. Add 400 µL of ddH_2O to the filter side of each Microcon 100 concentrator.

13. Spin again at a setting of 3000 X g for 5 minutes and discard the filtrate cups. (Note: additional spin time and/or higher speed, up to 4500 X g, may be required to filter the entire volume.)

14. Add 60 µL of filtered ddH_2O to the filter side of each Microcon 100 concentrator and place a retentate cup on the top of each concentrator.

15. Briefly vortex the Microcon 100 concentrators with the retentate cups pointing upward.

16. Invert each concentrator with its retentate cup and spin in a microcentrifuge at a setting of 10000 X g for 3 minutes.

17. Discard the concentrators. Using a pipetter, measure the volume of the retentate. Add ddH_2O if necessary to bring volume to 60 µL. Cap the retentate cups.

18. The samples can then be amplified using the following dilutions in ddH_2O as general guides:

    - Heavy blood stains on cotton sheeting, FTA paper, or filter paper: 1:50
    - Light blood stains on cotton sheeting, FTA paper, or filter paper: 1:10
    - Liquid blood: 1:50
    - Any blood stain on gauze: 1:10
    - Half a buccal swab: 1:50

3.3 Procedure for DNA Extraction from Calcified Tissue

3.3.1 Initial Assessment of Teeth

Prior to characterization of DNA from teeth, they should be examined by a forensic dentist, anthropologist, or pathologist. An odontology report should be submitted to the FBI Laboratory for cases to be analyzed for the National Missing Persons DNA Database. Photograph teeth prior to extraction. Samples may be cleaned using ddH_2O, 5% Terg-a-zyme, or 1% SDS prior to extraction at the discretion of the Examiner and Biologist based on the nature of the evidence.

If several teeth are available for analysis, the order of preference for tooth selection for DNA extraction is as follows:

a) Non-restored molar
b) Non-restored premolar
c) Non-restored canine
d) Non-restored anterior tooth
d) Restored molar
e) Restored premolar
f) Restored canine
g) Restored anterior tooth

3.3.2 Initial Assessment of Bone

Prior to mtDNA analysis of bone samples, the bone should be examined by a forensic pathologist and/or a qualified forensic anthropologist and the results submitted to the FBI Laboratory. If soft tissue is present, DNA may be extracted from the soft tissue using the tissue extraction protocol. DNA may be extracted from bone marrow also using the tissue extraction protocol. Photograph bones prior to extraction. Samples may be cleaned using ddH_2O, 5% Terg-a-zyme, or 1% SDS prior to extraction at the discretion of the Examiner and Biologist based on the nature of the evidence.

If several bones are available for analysis, the order of preference for bone selection for DNA extraction is as follows:

a) Long bone (cut section so as to avoid cutting bone in two)
b) Rib (mid-section)
c) Other (determined by Examiner)

For bones or teeth whose sequences will be added to the National Missing Persons DNA Database, a portion of the DNA extract will be sent to DNA Analysis Unit I.

3.3.3 Preparation of Reagent Blank and Cylinder

1. Thoroughly clean the cylinder, metal end plugs and impactor bar with either DNA-OFF or a fresh 10% bleach solution. Rinse with ddH_2O. Expose the cylinder, end plugs and impactor to UV light for a minimum of 15 minutes.

2. Prepare the reagent blank by swabbing the inside of the cylinder, end plugs, and impactor bar with an appropriate number of sterile, dry cotton swabs (typically two or more).

3. Place a portion of one swab in a 2 mL extraction tube. Add 1.6 mL of 0.5 M ethylenediamine tetraacetic disodium salt **(**EDTA). Incubate tubes with agitation (on Nutator) up to 24 hours at room temperature.

3.3.4 Cutting a Tooth

Note: Wear protective eyewear for these steps.

1. Using a rotary tool with an emery disk, sand the outer surface of the tooth so that the outer surface appears free of dirt and debris. Discard the emery disk.

2. Using the rotary tool with a separating disk, cut the tooth horizontally at the cemento-enamel junction being careful to avoid any restorations that may be present. (The tooth may be placed into a vise to hold it.) Make an additional horizontal cut in the tooth slightly apical to the original cut so that a small cross section of root can be separated from the remainder of the tooth. Alternatively, the entire root may be used. Discard the separating disk.

3. Examine tooth for the possible presence of soft tissue. If any is present, remove it from the pulp chamber and place in a sterile 2 mL extraction tube and proceed to Section 3.2, DNA Extraction for Body Fluids and Tissues.

4. Proceed to Section 3.3.6 for pulverization of tooth.

3.3.5 Cutting a Bone

Note: Wear protective eyewear for these steps.

1. Using a rotary tool with an emery disk, sand the outer surface of the bone so that the outer surface appears free of dirt and debris. If bone has been cut previous to submission or is hollow, also sand interior surface. Discard the emery disk.

2. Cut the bone using a rotary tool with a separating disk to a piece measuring approximately 1-2 cm x 1-2 cm. Discard the separating disk. Proceed to Section 3.3.6 for pulverization of bone.

3. If there is material within the marrow space, remove it with forceps and place the material in a sterile 2 mL extraction tube and proceed to Section 3.2, DNA Extraction for Body Fluids and Tissues.

3.3.6 Pulverization of Teeth And Bones

Teeth and bones can be pulverized using the SPEX 6750 or 6800 Freezer Mill.

1. Assemble the cleaned sample vial (either the 6751 or 6801) by inserting one end-plug into the cylinder. End-plugs should be inserted concave side in. Place the impactor bar in the sample cylinder along with the cut portion of the bone or tooth. Insert the second end-plug.

3.3.6.1 SPEX 6750 Freezer/Mill

1. Keeping the top of the 6750 Freezer Mill open, fill it with liquid nitrogen to indicated line inside (approximately 5 L).

   Chemical Hazard: Liquid nitrogen can be hazardous. Use cryogenic gloves, appropriate clothing and protective eyewear when handling liquid nitrogen. Be careful not to expose face or hands to liquid nitrogen splashes.

2. Set the freezer mill program and run it as follows:

   T1: 5.0
   T2: 2.0
   T3: 0.1
   Rate: 15

   Refer to the user's manual for details on programming the freezer mill. Do not open the lid of the freezer mill during grinding.

3. When the grinding cycle is finished, open the lid, remove the cylinder with the Extractor and Cylinder Opener, and inspect the sample. If sample is not sufficiently pulverized, reinsert for additional grinding. Addition of liquid nitrogen may be necessary.

4. Once the cylinder containing the sample has been removed from the freezer mill, the cylinder may be left to warm to room temperature before removing the sample or the cylinder may be opened while still cold. Place the sample vial in the sample rack. Align

the pins on the sample vial end-plug with the slots on the Extractor. Turn the Extractor handle clockwise to release the end-plug.

5. Remove the impactor bar and empty the pulverized sample onto a new piece of weigh paper. Carefully pour a portion of the pulverized sample into the sterile 2 mL extraction tube. Store remaining powder at 4°C.

6. Proceed to Section 3.3.7 to continue extraction.

3.3.6.2 SPEX 6800 Freezer/Mill

1. Turn on the 6800 Freezer Mill using the On/Off switch located on the lid of the freezer mill and ensure the mill is in the Program Mode.

2. Open the lid of the freezer mill using the Lid Up/Down switch located on the left rear corner of the lid. The lid is raised and lowered by an electrical drive. Hold the switch down until the lid is up.

3. Place the sample cylinder into the freezer mill by opening the coil gate. Open the coil gate by turning the handle counterclockwise to align it with the slot in the gate. Lower the gate until it rests on the stop below its hinge. The coil gate will hold one 6801 sample vial or four 6751 vials.

4. To close the gate, make sure the handle is aligned with the slot in the gate and raise the gate past the handle. Turn the handle so it is positioned at right angles to the slot. Push the handle down against the gate to lock it.

5. Close the lid of the freezer mill by moving the Lid Up/Down switch toward the front of the mill. Hold the switch down until the lid closes.

   Note: If the lid is not fully closed the LED display will read "LID" and the controls will not function.

6. Fill the freezer mill with liquid nitrogen. The liquid nitrogen level is electronically monitored by the Level Detector. To determine the amount of liquid nitrogen in the mill, press keys 2 and 3 on the keypad simultaneously. If all four lights on the display are lit, the mill is full. Filling requires about 20 L.

   Chemical Hazard: Liquid nitrogen can be hazardous. Use cryogenic gloves, appropriate clothing and protective eyewear when handling liquid nitrogen. Be careful not to expose face or hands to liquid nitrogen splashes.

Note: The mill should not be used if only two of the lights are displayed. Check the liquid nitrogen level before starting the grinding program. Each hour of operation consumes 4-5 L of liquid nitrogen.

7. Set the run parameters as follows:

   Press "Cycle" and enter "1". To store this setting press "ENT" and "0" at the same time.

   All settings inputted are only stored during the run. Turning off the freezer mill deletes all settings.

   Set the impactor rate by pressing the up arrow to a setting of 15. To store this setting press "ENT" and "0" at the same time. The impactor frequency can be increased during the run by pressing the increment key. When the grinding cycle is completed the LED display will blink "END" and return to the Program Mode. The freezer mill may be stopped during a run by pressing the STP key. The LED display will blink "END" and then return to Program Mode.

8. When the grinding cycle is finished, open the freezer mill lid and coil gate.

9. To remove the sample vial, slip the wide end of the Extractor body over the exposed end plug and turn the knob of the Extractor clockwise to thread the screw into the end-plug.

10. Place the sample vial in the sample rack. Align the pins on the sample vial end-plug with the slots on the Extractor. Turn the Extractor handle clockwise to release the end-plug.

11. Remove the impactor and empty the pulverized sample onto a new piece of weigh paper. Carefully pour the pulverized sample into a sterile 2 mL extraction tube. Store powder at $4^{o}C$.

12. Proceed to Section 3.3.7 to continue extraction.

3.3.7 Extraction of DNA

1. Carefully pour a portion of the pulverized sample into a sterile 2 mL extraction tube. Fill the tube to approximately the top of its base (approximately 1/4 full, 0.1 g). Depending upon the condition of the bone, at the discretion of the Examiner and Biologist, more than one tube of powder may be used for extraction.

2. Add 1.6 mL 0.5 M EDTA to each tube. Close tubes carefully and seal tops with Parafilm if desired. (Be certain that tops of flip top tubes close snugly -- some tubes have defective caps and do not close tightly.)

3. Vortex gently to suspend powder.

4. Incubate tubes with agitation (on Nutator) up to 24 hours at room temperature (8-16 hours is optimal).

5. Centrifuge tubes one minute at 8000 X g to pellet powder.

6. Remove supernatant.

7. Wash RB swab and powder by adding 1 mL ddH_20 to each tube. Close lid, vortex briefly. Centrifuge tubes one minute at 8000 X g to pellet powder. Remove supernatant.

8. Repeat water wash step two more times for a total of three washes.

9. Add 300 µL of prepared SEB (Section 10.1) and 2 µL of 600 U/mL of proteinase K to RB and powder. Vortex briefly.

10. Incubate powder in SEB with agitation (on Nutator) up to overnight (6-8 hours is optimal) in a 56^{o}C incubator.

11. Pulse-spin tubes. Place the RB swab in a filter basket, insert basket into extraction tube, and pulse-spin. Discard RB swab.

12. Add 300 µL AL buffer that has been exposed to UV light in a crosslinker for a minimum of 15 minutes to each sample and RB tubes. The solution may appear to be very viscous. Vortex to mix. Incubate tubes 10 minutes at 70^{o}C. Centrifuge briefly.

13. Add 400 µL ethanol that has been exposed to UV light in a crosslinker for a minimum of 15 minutes to each tube. Vortex to mix. Centrifuge briefly.

14. Carefully transfer 500 µL mixture (including any precipitate) directly to QIAamp spin column that has been exposed to UV light in a crosslinker for a minimum of 15 minutes. Avoid touching pipette tip to lip of column and do not touch pipette tip to filter in column. Centrifuge column for one minute at 8000 X g. Discard the flow-through and return the spin column to the collection tube.

15. Transfer remaining 500 µL mixture (including any precipitate) directly to QIAamp spin column. Avoid touching pipette tip to lip of column and do not touch pipette tip to filter in column. Centrifuge column for one minute at 8000 X g. Transfer spin column to new collection tube.

16. Add 500 µL AW1 buffer that has been exposed to UV light in a crosslinker for a minimum of 15 minutes directly to QIAamp spin column. Centrifuge column for one minute at 8000 x g. Transfer spin column to new collection tube.

17. Add 500 µL AW2 buffer that has been exposed to UV light in a crosslinker for a minimum of 15 minutes directly to QIAamp spin column. Centrifuge column for three minutes at 13000-14000 X g.

18. Discard the flow through and return the spin column to the collection tube. Centrifuge column for one minute at 13000-14000 X g.

19. Transfer spin column to sterile 2 mL tube for elution of DNA. Add sufficient ddH_2O for recovery of 60 µL. Incubate at 70°C for five minutes. Centrifuge column for one minute at 8000 X g to collect DNA extract. Using a pipette, measure the volume of the retentate. Add ddH_2O if necessary to bring volume to 60 µL.

20. The samples can then be amplified. Store extracted DNA at 4°C.

4 Procedure for DNA Amplification

The primers and primer sets used for mtDNA amplification are listed below. Mitochondrial DNA primers are named for the particular strand of mtDNA from which they are derived and designated with the number of the terminal 3' base of the primer. The convention is "L" for light strand and "H" for heavy strand, followed by the number of that base as originally published (see Anderson, et al., 1981).

4.1 Amplification primers

Standard Primers - HV1

A1: (L 15997) 5'-CAC CAT TAG CAC CCA AAG CT-3'

B2: (H 16237) 5'-GGC TTT GGA GTT GCA GTT GAT-3'

A2: (L 16159) 5'-TAC TTG ACC ACC TGT AGT AC-3'

B1: (H 16391) 5'-GAG GAT GGT GGT CAA GGG AC-3'

Standard Primers- HV2

C1: (L 048) 5'-CTC ACG GGA GCT CTC CAT GC-3'

D2: (H 285) 5'-GGG GTT TGG TGG AAA TTT TTT G-3'

C2: (L 177) 5'-TTA TTT ATC GCA CCT ACG TTC AAT-3'

D1: (H 409) 5'-CTG TTA AAA GTG CAT ACC GCC-3'

Whole Control Region Primers

A1: (L 15997) 5'-CAC CAT TAG CAC CCA AAG CT-3'

617 (H 617) 5'-TGA TGT GAG CCC GTC TAA AC-3'

Mini-Primers

1A (L 16055) 5'-GAA GCA GAT TTG GGT ACC AC-3'

1B (H 16142) 5'-ATG TAC TAC AGG TGG TCA AG-3'

2A (L16131) 5'-CAC CAT GAA TAT TGT ACG GT-3'

2B (H 16218) 5'-TGT GTG ATA GTT GAG GGT TG-3'

3A (L 16209) 5'-CCC CAT GCT TAC AAG CAA GT-3'

3B (H 16303) 5'-TGG CTT TAT GTA CTA TGT AC-3'

4A (L 16287) 5'-CAC TAG GAT ACC AAC AAA CC-3'

4B (H 16356) 5'-GTC ATC CAT GGG GAC GAG AA-3'

5A (L 16347) 5'-CGT ACA TAG CAC ATT ACA GT-3'

5B (H 16410) 5'-GCG GGA TAT TGA TTT CAC GG-3'

For routine reference samples consisting of blood and/or saliva, and for questioned samples where a potentially abundant amount of mtDNA template is present, HV1 and HV2, or the whole control region (WCR) can be amplified using the primer sets listed below.

For questioned samples such as hairs without tissue, or degraded bone and teeth where a potentially lower amount of mtDNA template is expected, HV1A, HV1B, HV2A, and HV2B should be amplified using the primer sets listed below. For questioned samples where extremely degraded DNA is expected, MPS1-MPS5 can be amplified using the mini-primer sets (MPS) listed

below.

Region	Primer Set
HV1	A1, B1
HV2	C1, D1
HV1A	A1, B2
HV1B	A2, B1
HV2A	C1, D2
HV2B	C2, D1
WCR	A1, 617
MPS1	1A, 1B
MPS2	2A, 2B
MPS3	3A, 3B
MPS4	4A, 4B
MPS5	5A, 5B

4.1.1 Amplification by PCR with Standard Primer Sets

1. Thermal cycler parameters for the ABI Model 9700 thermal cycler are as follows:

   95°C for 9 minutes

   32 or 36* cycles: 95°C for 10 seconds
   60°C for 30 seconds
   72°C for 30 seconds

   4°C hold indefinitely

   *In general, use 36 cycles for samples containing low DNA quantities (typically, hair, bones, and teeth), and 32 cycles for samples containing higher DNA quantities (typically

bodily fluids).

2. Use 10 µL of the DNA extract or its appropriate dilution.

3. Label reaction tubes with the appropriate designation to indicate the region amplified and sample type (negative control, reagent blank, sample, and positive control).

4. Prepare an amplification master mix for each primer set, taking into account that a negative control, positive control, and reagent blank will be amplified with each sample. Typically, a 5 sample master mix is sufficient.

(All volumes are in µL)

Component	1 Sample	5 Samples
ddH_2O	6.0	30.0
GeneAmp® 10X PCR Buffer	2.5	12.5
BSA (1.6 µg/µL)	2.5	12.5
dNTP mix	2.0	10.0
primer (L) 30 µM	0.5	2.5
primer (H) 30 µM	0.5	2.5
AmpliTaq Gold®DNA Polymerase (5U/µL)	1.0	5.0
TOTALS	15.0	75.0

5. Aliquot 15 µL of the appropriate master mix into each MicroAmp reaction tube.

6. Add 10 µL of ddH_2O to each negative control tube and cap each tube.

Note: Pipette tips are changed between each sample.

7. Add 10 µL of the reagent blank to each RB tube and cap each tube.

8. Add 10 µL of the sample extract to each sample tube and cap each tube.

9. Add 10 µL of positive control DNA (total 100 pg) to each positive control tube and cap each tube.

10. Carry the tubes to the thermal cycler.

Note: You are now moving into post-amplification space.

11. Place the reaction tubes in a MicroAmp retainer rack in the thermal cycler. Start the

appropriate thermal cycler amplification program.

12. The amplification program is completed in approximately 2 hours. Store the samples at 4°C in a space dedicated to post-amplification processing. Alternatively, samples may be stored at -20 °C. Samples can then be purified using either Microcon 100 filtration in Section 4.2 or ExoSAP-IT in Section 4.3.

4.1.2 Amplification by PCR with Whole Control Region (WCR) Primers

1. Thermal cycler parameters for the ABI Model 9700 thermal cycler are as follows:

   95°C for 9 minutes

   32 cycles: 95°C for 10 seconds
   53°C for 30 seconds
   72°C for 30 seconds

   72°C for 10 minutes

   4°C hold indefinitely

2. Use 20 µL of the extract or its appropriate dilution.

3. Label reaction tubes with the appropriate designation to indicate the sample type (negative control, reagent blank, sample identifier, and positive control).

4. Prepare an amplification master mix for the WCR primer set, taking into account that a negative control, positive control, and reagent blank will be amplified with each sample. Typically, a 5 sample master mix is sufficient. (All volumes are in µL)

Component	1 Sample	5 Samples
ddH_2O	12.0	60.0
GeneAmp® 10X PCR Buffer	5.0	25.0
BSA (1.6 µg/µL)	5.0	25.0
dNTP mix	4.0	20.0
A1 primer 30 µM	1.0	5.0
617 primer 30 µM	1.0	5.0
AmpliTaq Gold® DNA Polymerase (5U/µL)	2.0	10.0
TOTALS	30.0	150

5. Aliquot 30 µL of the appropriate master mix into each MicroAmp reaction tube.

6. Add 20 µL of ddH_2O to each negative control tube and cap each tube.

Note: Pipette tips are changed between each sample.

7. Add 20 µL of the reagent blank to each RB tube and cap each tube.

8. Add 20 µL of the sample extract to each sample tube and cap each tube.

9. Add 20 µL of positive control DNA (total 200 pg) to each positive control tube and cap each tube.

10. Carry the tubes to the thermal cycler.

Note: You are now moving into post-amplification space.

11. Place the reaction tubes in a MicroAmp retainer rack in the thermal cycler. Start the appropriate thermal cycler amplification program.

12. The amplification program is completed in approximately 2 hours. Store the samples at 4°C in a space dedicated to post-amplification processing. Alternatively, samples may be stored at -20 °C. Samples can then be purified using either Microcon 100 filtration in Section 4.2 or ExoSAP-IT in Section 4.3.

4.1.3 Amplification by PCR with Mini-Primer Sets

1. Thermal cycler parameters for the ABI Model 9700 thermal cycler are as follows:

95°C for 12 minutes

36 cycles: 95°C for 15 seconds
56°C for 30 seconds
72°C for 45 seconds

4°C hold indefinitely

2. Use 10 µL of the extract or its appropriate dilution.

3. Label reaction tubes with the appropriate designation to indicate the region amplified and sample type (negative control, reagent blank, sample identifier, and positive control).
4. Prepare an amplification master mix for each primer set, taking into account that a negative

control, positive control, and reagent blank will be amplified with each sample. Typically, a 5 sample master mix is sufficient.

(All volumes are in µL)

Component	1 Sample	5 Samples
ddH_2O	6.0	30.0
GeneAmp® 10X PCR Buffer	2.5	12.5
BSA (1.6 µg/µL)	2.5	12.5
dNTP mix	2.0	10.0
primer (L) 10 µM	0.5	2.5
primer (H) 10 µM	0.5	2.5
AmpliTaq Gold® DNA Polymerase (5U/µL)	1.0	5.0
TOTALS	15.0	75.0

5. Aliquot 15 µL of the appropriate master mix into each MicroAmp reaction tube.

6. Add 10 µL of ddH_2O to each negative control tube and cap each tube.

   Note: Pipette tips are changed between each sample.

7. Add 10 µL of the reagent blank to each RB tube and cap each tube.

8. Add 10 µL of the sample extract to each sample tube and cap each tube.

9. Add 10 µL of positive control DNA (total 100 pg) to each positive control tube and cap each tube.

10. Carry the tubes to the thermal cycler.

    Note: You are now moving into post-amplification space.

11. Place the reaction tubes in a MicroAmp retainer rack in the thermal cycler. Start the appropriate thermal cycler amplification program.

12. The amplification program is completed in approximately 2 hours. Store the samples at 4°C in a space dedicated to post-amplification processing. Alternatively, samples may be stored at -20 °C. Samples can then be purified using either Microcon 100 filtration in Section 4.2 or ExoSAP-IT in Section 4.3.

4.2 Amplicon Purification by Microcon 100 Filtration

1. Assemble and label a Microcon 100 unit for each sample. Add 400 µL of ddH_2O to the filter side of each Microcon 100 concentrator.

2. Add the entire amplified product from each MicroAmp reaction tube to the appropriately labeled concentrator.

3. Spin the Microcon 100 concentrators for 5 minutes at a setting of 3000 X g. (Note: additional spin time and/or higher speed, up to 4500 X g, may be required to filter the entire volume.)

4. Discard the wash and return the filtrate cups to the concentrators.

5. Add 400 µL of filtered ddH_2O to the filter side of each Microcon 100 concentrator.

6. Spin again at a setting of 3000 X g for about 5 minutes and discard the filtrate cups. (Note: additional spin time and/or higher speed, up to 4500 X g, may be required to filter the entire volume.)

7. Add 40 µL of filtered ddH_2O to the filter side of each Microcon 100 concentrator and place a retentate cup on the top of each concentrator.

8. Briefly vortex the Microcon 100 concentrators with the retentate cups pointing upward.

9. Invert each concentrator with its retentate cup and spin in a microcentrifuge at a setting of 10000 X g for 3 minutes.

10. Discard the concentrators. Cap the retentate cups containing the purified amplicons for quantification by capillary electrophoresis and sequencing. Store samples at 4^oC until ready for quantification and sequencing.

4.3 Amplicon Purification by ExoSAP-IT

1. Following amplification with standard or mini-primer sets, add 5 µL ExoSAP-IT cocktail to each PCR reaction tube, yielding a total volume of 30 µL per tube. Following amplification with whole control regions primers, add 10 µL ExoSAP-IT cocktail to each PCR reaction tube, yielding a total volume of 60 µL.

2. Incubate PCR tubes in thermal cycler programmed as follows:

   37^oC for 15 minutes

80°C for 15 minutes
4°C hold indefinitely

3. Store at 4°C until ready for quantification and sequencing. Alternatively, samples may be stored at -20 °C.

4. Prior to quantification, the volume of each PCR reaction should be measured with a pipetter, and if necessary, add an appropriate amount of sterile ddiH20 to bring the volume up to the expected amount (30 uL for amplifications with standard or mini-primer sets, or 60 uL for amplifications with whole control region primers).

NOTE: If the volume of the amplification is not adjusted prior to quantification, the volume may not be adjusted after quantification.

5 Procedure for Post-Amplification Quantification by Capillary Electrophoresis

Following post-amplification purification, the amplified mtDNA samples are quantified by capillary electrophoresis prior to sequencing using the Agilent Technologies 2100 Bioanalyzer and the DNA 1000 or 7500 Series II LabChip kit. The Agilent Technologies 2100 Bioanalyzer is operated, and the data collected and analyzed using the 2100 Expert software (Rev. B.02.03) supplied by Agilent (Section 5.1.3). For further details on particular aspects of the instruments or the software, refer to the relevant Instrument Manuals and Software Reference Manuals.

5.1 Procedure for using the Agilent Technologies 2100 Bioanalyzer

Note: The DNA 1000 Series II LabChip kit (Section 5.1.1) is typically used for samples amplified with the standard primer sets as well as the mini-primer sets. The DNA 7500 Series II LabChip kit (section 5.1.2) is typically used for samples amplified with the whole control region primers.

5.1.1 Using The DNA 1000 Series II Labchip Kit

CAUTION: Kit components contain dimethyl sulfoxide (DMSO). Because the dye binds to nucleic acids, it should be treated as a potential mutagen. Wear gloves and protective eyewear when handling kit components.

5.1.1.1 Preparing the DNA 1000 Gel-Dye Mix

1. Add 25 µL of dye into a DNA gel matrix vial.

2. Cap the tube and vortex well.

3. Transfer the gel-dye mix to the top receptacle of the spin filter provided in the kit.

4. Centrifuge the spin filter for 15 minutes at approximately 2240 X g.

5. Label the tube and include the date of preparation.

Note: The gel-dye mix must be stored at 4°C at the end of each day's use. It must be protected from light at all times, and equilibrated to room temperature before use. Use the gel-dye mix within 4 weeks of preparation.

5.1.1.2 Priming the DNA 1000 LabChip

Note: Allow all reagents to warm to room temperature before use. This can be accomplished by placing the chip reagent box inside a closed drawer for at least 30 minutes. If the reagents are not properly warmed, some fragments on the ladder may not electrophorese in the allotted time. The chip should be run within 5 minutes of preparation to avoid failure due to evaporation. Addition of air bubbles should be minimized by avoiding "blowing out" pipette tips. Turn on the appropriate Bioanalyzer and computer during this step.

1. Add 9 µL prepared gel-dye mix to the well marked ©.

2. Place the chip into the Chip Priming Station (marked for DNA 1000 use) and ensure syringe plunger is at 1 mL.

3. Close the priming station and press the syringe plunger until it is held by the syringe clip in the lowest position.

4. Wait exactly 60 seconds, then release the clip mechanism and pull back the syringe plunger to the 1 mL position.

5. Open the Chip Priming Station and remove chip.

6. Add 9 µL prepared gel-dye mix to each of the 2 wells marked "G".

7. Add 1 µL of the DNA 1000 ladder (yellow tube) to the well marked with the ladder symbol.

8. Add 5 µL of the DNA 1000 markers (green tube) to the well marked with the ladder symbol.

5.1.1.3 Loading the DNA 1000 LabChip

1. Add 5 µL of the DNA 1000 markers (green tube) to each of the 12 sample wells.

2. Add 1 µL sample to the appropriate sample well.

3. Add 1 µL DNA 1000 markers (green tube) or ddH_2O to any unused sample wells. (Alternatively, in step 1, 6 µL of the DNA 1000 markers can be added to the unused sample wells.)

4. Place the chip into the adapter of the vortex mixer and vortex for exactly 60 seconds at 2400 rpm.

5.1.2 Using the DNA 7500 Series II LabChip Kit

5.1.2.1 Preparing the DNA 7500 Gel-Dye Mix

1. Add 25 µL of dye into a DNA gel matrix vial.

2. Cap the tube and vortex well.

3. Transfer the gel-dye mix to the top receptacle of the spin filter provided in the kit.

4. Centrifuge the spin filter for 10 minutes at 1500 X g.

5. Label the tube and include the date of preparation.

Note: The gel-dye mix should be stored at 4°C at the end of each day's use. It should be protected from light at all times, and equilibrated to room temperature before use. Use the gel-dye mix within 4 weeks of preparation.

5.1.2.2 Priming the DNA 7500 LabChip

Note: Allow all reagents to warm to room temperature before use. This can be accomplished by placing the chip reagent box inside a closed drawer for <u>at least</u> 30 minutes. If the reagents are not properly warmed, some fragments on the ladder may not electrophorese in the allotted time and the run should be aborted. The chip should be run within 5 minutes of preparation to avoid failure due to evaporation. Addition of air bubbles should be minimized by avoiding "blowing out" pipette tips. The appropriate Bioanalyzer and computer should be turned on during this step.

1. Add 9 µL prepared gel-dye mix to the well marked ©.

2. Place the chip into the Chip Priming Station (marked for DNA 7500 use) and ensure syringe plunger is at 1 mL.

3. Close the priming station and press the syringe plunger until it is held by the syringe clip in the highest position.

4. Wait exactly 30 seconds, then release the clip mechanism and pull back the syringe plunger to the 1 mL position.

5. Open the Chip Priming Station and remove chip.

6. Add 9 µL prepared gel-dye mix to each of the 2 wells marked "G".

7. Add 1 µL of the DNA 7500 ladder (yellow tube) to the well marked with the ladder symbol.

8. Add 5 µL DNA of the 7500 markers (green tube) to the well marked with the ladder symbol.

5.1.2.3 Loading the DNA 7500 LabChip

1. Add 5 µL of the DNA 7500 markers (green tube) to each of the 12 sample wells.

2. Add 1 µL sample to the appropriate sample well.

3. Add 1 µL DNA 7500 markers (green tube) or ddH_2O to any unused sample wells. (Alternatively, in step 1, 6 µL of the DNA 7500 markers can be added to the unused sample wells.)

4. Place the chip into the adapter of the vortex mixer and vortex for exactly 60 seconds at 2400 rpm.

5.1.3 Running the Agilent 2100 Bioanalyzer

Each Agilent 2100 Bioanalyzer is controlled by a computer with the 2100 Expert Software (Rev. B.02.03) installed.

5.1.3.1 Running the Agilent 2100 Bioanalyzer Using the 2100 Expert Software

1. Turn on the appropriate Bioanalyzer and then the computer. **To minimize evaporation, it is recommended that this step be done prior to priming the chip.** Start the software by

double-clicking on the 2100 expert icon on the computer desktop. Click on the Instrument context and select the appropriate Bioanalyzer unit.

2. Insert the chip into the Agilent 2100 Bioanalyzer and close the lid. The software should now display a chip icon at the upper left of the screen.

3. Select the DNA 1000 assay (or DNA 7500) by selecting DNA 1000 II (or DNA 7500 II) from the Assay Selection button on the upper right hand section of the Instrument screen.

4. To name the file, enter the Laboratory and specimen numbers in the file prefix box in the Destination section of the Instrument screen.

5. Click the Start button on the upper right hand section of the Instrument tab screen (underneath the Assay Selection button).

6. Fill out the Sample Information table by clicking on the Data File: name (in blue) or by clicking on the Data and Assay icon in the Contexts panel. When finished entering sample information, click on the Instrument context and click yes to save to the chip summary (this will return you to the Instrument screen as well).

7. When the run is complete, the Bioanalyzer will chime. Clean the electrodes by adding 350 µL ddH_2O to the electrode cleaner. Place the cleaner in the Bioanalyzer and close the lid for 10 seconds. Open the lid, remove the electrode cleaner, and wait another 10 seconds. Close the lid.

8. The 2100 Expert software calculates the concentration of the sample. Concentrations for multiple peaks may be added together to account for length differences seen in particular samples. If this concentration exceeds 50 ng/µL, the sample should be diluted and rerun.

6 Procedure for Cycle Sequencing

Samples are subjected to cycle sequencing, gel filtration, and drying in preparation for electrophoresis using the ABI Prism 3130 Genetic Analyzer.

6.1 Cycle Sequencing Reaction

Cycle sequencing is performed using either the ABI Prism dRhodamine Terminator Cycle Sequencing Ready Reaction Kit or the ABI Prism BigDye Terminator v1.1 Cycle Sequencing Kit. The reaction is carried out by combining the sequencing reaction mix, mtDNA template and a single sequencing primer in a 0.2 mL microcentrifuge tube. The reaction mixture is subjected to a series of controlled temperature changes in a GeneAmp PCR system 9700 thermal cycler. The

amount of mtDNA template used for sequencing should be approximately 10 ng. However, this amount is not absolute, and lesser or greater amounts of template can be used. A minimum of 7 ng of amplified DNA template from the sample and positive control must be used for cycle sequencing. Appropriate rounding may be used for the minimum calculation.

For samples amplified with the standard primers or mini-primer sets, the primers used for sequencing are identical to those used in amplification (see Section 5). The primers A4 and B4 are used on samples which contain a homopolymeric region from positions 16184-16194. They are designed to bind near the homopolymeric region and prime the sequencing reaction on their respective strands. Primer A4 is used to sequence the HV1B region. Primer B4 is used to sequence the HV1A region. Primer 317f can be used to sequence samples which exhibit significant length heteroplasmy in the 303-310 region.

A4: (L 16209) 5'-CCC CAT GCT TAC AAG CAA GT-3' (HV1B)

B4: (H 16164) 5'-TTT GAT GTG GAT TGG GTT T-3' (HV1A)

317: 5'-CCC CCC CTC CCC CCG C-3' (HV2B)

For samples amplified with the whole control region primer set, the following primers are used for sequencing, although these primers may also be used for sequencing samples amplified with the standard primer sets when necessary. Primer 557 can be used to sequence samples which exhibit significant length heteroplasmy in the 568-573 region.

L 15597 (A1): 5'-CAC CAT TAG CAC CCA AAG CT-3'

H 16164 (B4): 5'-TTT GAT GTG GAT TGG GTT T-3'

L 16209 (A4): 5'-CCC CAT GCT TAC AAG CAA GT-3'

L 16511: 5'-CCG ACA TCT GGT TCC TAC-3'

H 19: 5'-TCC CGT GAG TGG TTA ATA G-3'

H 296: 5'-RRR GGG GGG GTT TGG TG-3'

L 317: 5'-CCC CCC CTC CCC CCG C-3'

H 617: 5'-TGA TGT GAG CCC GTC TAA AC-3'

H 557: 5'-GGG GGG TGT CTT TGG GG-3'

1. To prepare purified mtDNA template, dilute if necessary (based upon the concentration determined by CE quantification) to approximately 10 ng mtDNA per 7 µL (full volume reaction) or 3.5 uL (half volume reaction) of added template. Label MicroAmp reaction tubes with the appropriate sample designation.

2. Add appropriate volumes of reagents into labeled MicroAmp tubes as indicated by the chart below. Add reagents in the order listed in the chart.

	Half volume reaction	Full volume Reaction
dRhodamine reaction mix	4.75 µL	9.5 µL
1 µM primer	1.75 µL	3.5 µL
Template (~10 ng)	3.5 µL	7 µL
Total volume	10 µL	20 µL

	Half volume reaction	Full volume reaction
***Diluted BigDye** sequencing mix	4.75 µL	9.5 µL
1 µM primer	1.75 µL	3.5 µL
Template (~10 ng)	3.5 µL	7 µL
Total volume	10 µL	20 µL

***Diluted BigDye sequencing mix is made immediately prior to use by combining BigDye sequencing mix and UV-treated dH_2O in a separate tube according to the table below.**

# tubes – half reaction	# tubes – full reaction	Vol BigDye (µl)	Vol dH_2O (µl)
4	-	18	3.4
8	4	36	6.75
12	-	54	10.1
16	8	72	13.5
20	-	90	16.9
24	12	108	20.25
28	-	126	23.6
32	16	144	27
36	-	162	30.4
40	20	180	33.75
44	-	198	37.1
48	24	216	40.5
52	-	234	43.9
56	28	252	47.25
60	-	270	50.6
64	32	288	54

NOTE: Any remaining diluted BigDye sequencing mix should be discarded.

NOTE: It is recommended that the half volume reaction procedure be used in most cases. If the concentration of the sample template is below 2.9 ng/uL, full volume reactions must be used.

3. Place the capped tubes into the GeneAmp 9700 thermal cycler, close and clamp the sample cover. Turn on the thermal cycler and run a program with the following parameters:

   96°C for 1 minute

   25 cycles: 96°C for 15 seconds*
   50°C for 1 second
   60°C for 1 minute

   4°C indefinite hold

   NOTE: Before pressing the final 'START' to begin running the thermal cycler program, ensure that 'Reaction Volume' under 'Select Method Options' is set to the appropriate volume for the size of the reaction (i.e. 10 uL for half volume reactions or 20 uL for full volume reactions). This option must be checked every time.

4. Samples are then prepared for electrophoresis using spin columns (Section 6.2).

   *If samples indicate a significant drop in signal intensity after the C-stretch when using primer H 617 (due to possible secondary structure), cycle sequencing may be repeated using a denaturation temperature of 98°C.

6.2 Preparing Samples for Electrophoresis Using Spin Columns

6.2.1 Centri-Sep Spin Column

Centri-Sep spin columns are prepared for removal of unincorporated fluorescent terminators from the completed sequencing reaction. Steps 1-4 may be completed prior to setting up the cycle sequencing reactions.

1. Gently tap the column to force the powder to the bottom of the column (the white filter is on the bottom).

2. Remove the top cap of the column and add 800 µL ddH_2O.

3. Replace the cap, invert, vortex at high speed, invert again, vortex, and tap firmly until the gel solution is thoroughly mixed and any bubbles have risen to the top.

4. Allow the gel to hydrate a minimum of 30 minutes at room temperature. Columns can be stored at 4^{o}C for up to a week. Let columns equilibrate to room temperature before use.

5. If it is necessary to remove the excess water from the spin columns, remove the top cap, then the bottom cap, and let the column drain in a rack. If drainage does not begin immediately, apply air pressure to the top of the column with a gloved thumb.

6. Place the appropriate number of wash tubes in a microcentrifuge, and place the drained spin columns into the wash tubes.

7. Spin at a setting of 1500 X g for 2 minutes.

8. While the columns are spinning, label the collection tubes with the appropriate sample designation.

9. Remove the columns from the wash tubes and place them into the sample collection tubes. Discard the wash. Note that at this point the columns must be used right away.

10. When the cycle sequencing is complete, remove the entire reaction mixture from each reaction tube and carefully load it onto the top of the gel material. It is important to dispense the sample onto the center of the gel without touching the sides of the column or disturbing the surface of the gel.

11. Place the collection tubes with spin columns in a centrifuge and spin at 1500 X g for 2 minutes.

12. Discard the spin columns.

13. Place the collection tubes containing purified sequencing fragments into a vacuum centrifuge and spin to dryness. Do not over dry. Typically, spinning 20-30 minutes accomplishes this. Dried samples can be stored at -20^{o}C for at least one week. Samples can then be subjected to electrophoresis (Section 7).

6.2.2 Centri-Sep 8 Strip Spin Columns

Centri-Sep 8 strip spin columns are prepared for removal of unincorporated fluorescent terminators from the completed sequencing reaction.

1. Remove the needed number of strips by cutting through the foil with scissors.

2. Open the well outlets on each strip by cutting through the integral bottom seal constriction with scissors.

3. Place the strips into the disposal plate and remove the top foil. Best results are obtained by placing the strips in the centermost wells of the disposal plate.

4. Remove the excess liquid by placing the strips in the disposal plate in a centrifuge and spin at 450 X g for 2 minutes.

5. Transfer the strips into the desired 96-well sequencing plate

6. Remove the entire completed cycle sequencing reaction mixture from each reaction tube and carefully load it onto the top of the gel material. It is important to dispense the sample onto the center of the gel without touching the sides of the column or disturbing the surface of the gel.

7. Place the sequencing plate in a centrifuge and spin at 450 X g for 2 minutes.

8. Discard the strips.

9. Place the sequencing plate containing purified sequencing fragments into a vacuum centrifuge and spin to dryness. Do not over dry. Typically, spinning 30-45 minutes accomplishes this. Dried samples can be stored at -20^{o}C for at least one week. Samples can then be subjected to electrophoresis (Section 7).

7 Procedure for Sequencing Using the ABI Prism 3130 Genetic Analyze

7.1 Setting Up the Instrument

CAUTION: Chemical Hazard. POP-6 may cause eye, skin and respiratory tract irritation. Wear the appropriate protective eyewear, clothing and gloves.

7.1.1 If the POP-6 polymer has been changed within the last 7 days AND the Genetic Analyzer (GA) buffer and water have been changed earlier in the day,

1. No maintenance is necessary. Proceed to Section 7.6, Sample Preparation.

7.1.2 If the POP-6 polymer has been changed within the last 7 days AND the GA buffer and water have NOT been changed earlier in the day,

1. Change the GA buffer in the Anode Buffer Reservoir (Section 7.5) and the Cathode Buffer Reservoir (Section 7.4). See Section 7.3 for preparation of the GA buffer.

2. Change the water in the Water Reservoirs (Section 7.4).

3. Records of the daily 3130 Genetic Analyzer maintenance shall be maintained.

7.1.3 If the POP-6 polymer has not been changed within the last 7 days AND the next bottle of polymer has the same lot number as the previous bottle,

1. Unscrew old bottle of polymer from machine, leaving cap with tubing in place. Rinse old bottle of polymer with water, completely deface label, and discard.

2. Remove cap from new bottle of polymer which has been allowed to equilibrate at room temperature for at least 30 minutes. Screw new bottle of polymer into cap with tubing in machine.

3. Remove Anode Buffer Reservoir ('shot glass') and refill with fresh GA buffer as indicated in Section 7.5. See Section 7.3 for preparation of the GA buffer.

4. Place folded Kimwipes into the overflow tray located below the Anode Buffer Reservoir.

5. Loosen the white spigots located in the upper polymer block. Screw a syringe full of clean ultra pure water into the front spigot, inject the water through the seal, remove the syringe and tighten the spigots. Catch rinse water with Kimwipes in overflow tray.

6. Run the REPLENISH POLYMER WIZARD in the 3130 Genetic Analyzer software and follow all prompts, including attach new polymer bottle, fill pump with polymer, remove bubbles, and fill array.

7. Replace Anode Buffer Reservoir filled with fresh GA buffer.

8. Change the GA buffer in the Cathode Buffer Reservoir and the water in the Water Reservoirs (Section 7.4). See Section 7.3 for preparation of the GA buffer.
9. Records of the weekly 3130 Genetic Analyzer maintenance shall be maintained.

7.1.4 If the POP-6 polymer has not been changed within the last 7 days AND the next bottle of polymer has a different lot number than the previous bottle,

1. Unscrew old bottle of polymer from machine, leaving cap with tubing in place. Rinse old bottle of polymer with water for use in step 5.

2 . Remove Anode Buffer Reservoir ('shot glass') and refill with fresh GA buffer as indicated in Section 7.5.

3. Place folded Kimwipes into the overflow tray located below the Anode Buffer Reservoir.

4. Loosen the white spigots located in the upper polymer block. Screw a syringe full of clean ultra pure water into the front spigot, inject the water through the seal, remove the syringe and tighten the spigots. Catch rinse water with Kimwipes in overflow tray.

5. Run the WATER WASH WIZARD and follow all prompts, including rinse out old polymer bottle, fill old polymer bottle with clean ultra pure water, attach old polymer bottle, fill pump with water, attach new polymer bottle, fill pump with polymer, and remove bubbles.

6. Replace Anode Buffer Reservoir filled with fresh GA buffer.

7. Change the GA buffer in the Cathode Buffer Reservoir and the water in the Water Reservoirs (Section 7.4). See Section 7.3 for preparation of the GA buffer.

8. Records of this 3130 Genetic Analyzer maintenance shall be maintained.

7.2 Installing a Capillary Array

1. Close the oven and instrument doors and then press the "Tray" button.

2. From the "Tool" menu, select "Install Capillary Array Wizard".

3. Follow the directions presented by the wizard to replace or install an array.

7.3 Preparing the Buffer

Note: Replenishing buffer and placing the plate on the instrument requires that the autosampler be in the forward position, with the capillary tips removed from the buffer solution. Do not leave the autosampler in this position for an extended period of time since the capillaries can become dry.

1. To prepare 1X Genetic Analyzer (GA) buffer, add 5 mL of 10X 3130 GA buffer to 45 mL of ddH_2O. Mix well.

2. Reservoirs containing 1X GA buffer and water are changed every day.

3. The 1X GA buffer can be stored at 4°C for up to two weeks.

7.4 Filling the Water and Cathode Buffer Reservoirs

1. Close the instrument doors.

2. Press the "Tray" button on the outside of the instrument to bring the autosampler to the forward position.

3. Wait until the autosampler has stopped moving and then open the instrument doors.

4. Remove the cathode buffer reservoir and ddH_2O reservoirs.

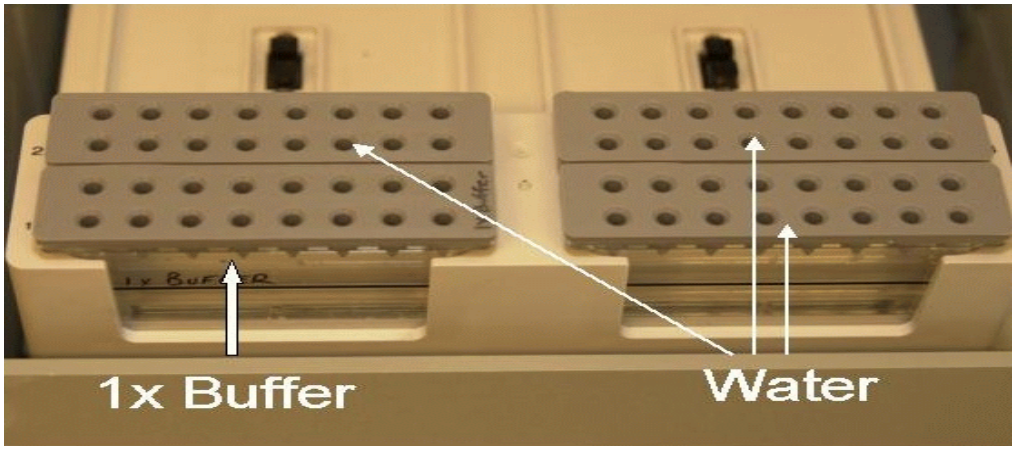

Figure 1: Buffer and water reservoirs on the ABI Prism 3130.

5. Discard the solutions into the sink and rinse out the reservoirs with ddH_2O.

6. Rinse the cathode reservoir with ddH_2O and then fill the reservoir to the fill line with 1X GA buffer (approximately 17 mL).

7. Fill the water reservoirs to the fill line with ddH_2O (approximately 17 mL).

8. Place a clean septa strip on each reservoir and dry the outside of the reservoirs using a lint-free wipe. Be sure that the septa fit snugly and are fitted flush on the tops of the reservoirs in order to prevent damaging the capillary tips.

9. Place the reservoirs into position on the autosampler as shown above in Figure 1.

7.5 Filling the Anode Buffer Reservoir

1. Remove the anode buffer reservoir by firmly pulling down and twisting counter-clockwise slowly.

2. Discard the used buffer appropriately by rinsing the buffer down the sink with water.

3. Clean and rinse the reservoir with ddH_2O and then rinse the reservoir with 1X GA buffer.

4. Fill the reservoir to the fill line with fresh 1X GA buffer after bubbles have been removed.

5. Put the anode buffer reservoir on the instrument. The meniscus should line up with the fill line.

7.6 Sample Preparation

7.6.1 For samples prepared using the Centri-Sep Spin Column

1. Resuspend the dried sample in 12 µL of Hi deionized formamide.

   CAUTION: Formamide is a suspected teratogen. Wear the appropriate protective eyewear, clothing and gloves.

2. Briefly centrifuge the tubes and transfer the samples to individual wells in a sample plate.

3. Secure a clean and dry septa strip on the sample plate. Ensure that the septa strip lies flat on the plate.

4. Briefly centrifuge the plate to ensure each sample is positioned at the bottom of its well.

7.6.2 For samples prepared using the Centri-Sep 8 Strip Spin Columns

1. Pipette 10 uL of Hi deionized formamide into each well in the sample plate containing a sample.

   CAUTION: Formamide is a suspected teratogen. Wear the appropriate protective eyewear, clothing and gloves.

2. Secure a clean and dry septa strip on the sample plate. Ensure that the septa strip lies flat on the plate.

3. Briefly centrifuge the plate to ensure each sample is positioned at the bottom of its well.

7.7 Plate Assembly

1. Place the sample plate into the plate and plate base.

2. Snap the plate retainer onto the plate base.

3. Check that the plate retainer holes are aligned with the holes in the septa strip.

   Note: Damage to the array tips will occur if the plate retainer and septa strip holes do not align correctly.

7.8 Placing the Plate onto the Autosampler

1. Press the "Tray" button on the outside of the instrument to bring the autosampler to the forward position.

2. Place the plate assembly on the autosampler. There is only one orientation for the plate, with the notched end of the plate base away from you.

3. Ensure the plate assembly fits flat in the autosampler. Failure to do so may allow the capillary tips to lift the plate assembly off of the autosampler.

4. When the plate is correctly positioned, the plate position indicator on the Plate View page changes from grey to yellow. Check to make sure that this has occurred.

5. Close the instrument doors. Closing the doors returns the autosampler to home position, placing the tips of the capillaries into the buffer.

7.9 Creating a Plate Record for DNA Sequencing Analysis

1. In the Navigation Pane on the left side of the screen of the Foundation Data Collection Version 3.0 software, expand the GA Instruments section by clicking on the "+" sign and highlight "Plate Manager".

2. In the Plate Manager page, select "New". The plate editor dialog box will appear.

3. Use the Plate Editor dialog box to name the plate. Include (at a minimum) the Laboratory Number and the sample designation in the plate name. Entering comments in the Description field is optional.

   Note: When naming the plate, use conventions specified by manufacturer. In

addition to spaces, the following symbols may <u>not</u> be used: \ / : * ? " < > |

4. Select the Sequencing Analysis application. Select the "96-well plate" type. Enter Owner Name and Operator Name.

5. When done, click "OK". The Plate Editor Spreadsheet will open.

   Note: The plate record can be created in Excel and imported for use as an alternative to steps 1-5 above.

7.10 Entering Sample Information

1. In the Plate Editor Spreadsheet, type the names of all of the samples in the Sample Name column.

   Note: When naming the samples, do not use spaces or the following symbols: \ / : * ? " < > |

2. Fill out the Comment field with the Laboratory number, sample information and the date. Choose "dna2" for the Results Group column. By default, the priority will be set to 100.

3. If using the dRhodamine Terminator Cycle Sequencing Ready Reaction Kit choose "mtDNA" in the Instrument Protocol 1 and Analysis Protocol 1 columns. Highlight the remaining samples and click on "Fill Down" to designate the Instrument and Protocol files for the remaining samples.

   If using the BigDye Terminator v1.1 Cycle Sequencing Kit choose "**BigDye**" in the Instrument Protocol 1 and Analysis Protocol 1 columns. Highlight the remaining samples and click on "Fill Down" to designate the Instrument and Protocol files for the remaining samples.

4. Check the spreadsheet for accuracy and click *"OK"*.

7.11 Linking a Plate to a Plate Record / Starting a Run

1. Expand the 3130 section in the Navigation Pane. Expand the Run Scheduler and click on "Plate View" to go to the Plate View page.

2. On the Plate View page:

   a) Find the Plate name by using the Find All button and highlighting the desired plate. Alternatively, an Advanced Search may be performed to find the desired plate.

b) Select the "Plate Position Indicator" that corresponds to the plate you are linking.

3. Verify that the plate has been linked. Once the plate has been linked:

a) The Run Instrument button on the Toolbar is enabled, meaning that the instrument is ready to run.

b) The Plate Position indicator for the linked plate will turn green.

4. Repeat steps 2 - 3 to link a second plate if applicable.

5. To begin the run, select the "green arrow" in the Toolbar.

6. Click on "Instrument Status" to monitor the run.

7.12 Storing a Capillary Array on the Instrument

1. Store the capillary array on the instrument when the capillary will not be used for less than one week.

2. The capillary tips should be submerged in 1X Genetic Analyzer buffer. Check that the level of 1X Genetic Analyzer buffer is at the fill line during the entire time of storage.

8 Procedure for Sequence Analysis, Comparison, and Interpretation

8.1 Data Analysis

After laboratory procedures are completed for a sample, the sequence data can be analyzed. Two strands (one from the forward primer and one from the reverse primer) must be used to edit sequence whenever possible. Single-stranded sequence can be used, but can be only edited toward ambiguity (the most conservative editing call) when there is a surrounding context for making the editing decision. See Section 8.2 for more detailed information.

8.1.1 Analysis of Data from the 3130 Instrument

The steps outlined below describe the general steps involved in analyzing sequencing runs. The user's manual for Sequence Analysis 5.2 contains details on using the software which may be used by the Examiner.

1. Launch Sequence Analysis. This can be accomplished by right-clicking on analysis files

within a run folder or from the Applied Biosystems menu on the Start menu. A username and password are required.

2. Add files to the Sample Manager window by selecting "Add Sample" from the File menu. Select desired run folder and choose desired analysis files. Click "Add Selected Samples" and "OK."

3. To view data from a sample file, click to check the "Show" box or highlight all samples and click on the "Show" button. The electropherogram may be viewed by clicking on the Electropherogram tab.

4. Display the raw data by clicking on the Raw tab. Note beginning and ending scan numbers by moving cursor to beginning and end of data peaks (the scan numbers should be similar for all capillaries within a run). Signal intensity may also be reviewed in the Raw tab.

5. Enter the beginning scan numbers in the "peak 1" and "start" columns. This assures that the first sequenced base is included.

6. Enter the ending scan numbers in the "stop" column. This will decrease the number of "N's" at the end of the run and focus the signal intensity calculations on the appropriate section. Note: A single "Peak 1 " and "Start," as well as a single "Stop" number may be used for all files within a run, as long as these numbers encompass all sequence data in each file.

7. Ensure the "BC" (BaseCaller) column is checked.

8. Click on the green arrow button to start the analysis.

9. When analysis is complete, save samples, close program and proceed to Section 8.1.2, "Identification of Polymorphic Sites in Sequences using Sequencher."

8.1.2 Identification of Polymorphic Sites in Sequences Using Sequencher

The steps outlined below describe the general steps involved in identifying polymorphic sites. The user's manual for Sequencher contains details on using the software which may be used by the Examiner.

1. Launch Gene Codes Sequencher software (version 4.7 for Windows).

2. Open "New Project" under the "File" pull-down menu.

3. Highlight the files you wish to import and drag them into the project window. Open each

sequence individually and delete unanalyzable sequence from the ends. Note which samples are unanalyzable (typically this includes RBs and NCs).

4. Highlight analyzable forward and reverse sequences for assembly and click on "Assemble Automatically". For analyzable RBs and NCs, the assembly parameters may need to be lowered in order to get the sequences to assemble. For samples and positive control samples, the default settings of 85 and 20 are typically sufficient.

5. View the contig produced. Check the direction of the sequence files by looking at the primers. If they are not in the appropriate orientation, go to the view menu and select "Reverse Comp".

6. Click on the "Bases" box to observe the nucleotide sequence, highlight a base in the consensus sequence and click on "Show Chromatograms" to observe the electropherograms. Delete the primer sequences.

   If the sequence is not of the requisite quality, for example, high background, poor peak resolution, or indicative of a mixture, the sequence will not be used. The sample may be re-extracted, re-amplified, re-cycle sequenced, or re-injected as appropriate.

7. Edit the sequences using information from all strands in the electropherogram window until the forward and reverse primer sequences are the same at all positions. Each base in the sequence must be manually reviewed for correct identification using the electropherogram windows. It is possible that ambiguous bases will remain after the sequence is fully edited. These ambiguities will be designated according to International Union of Pure and Applied Chemistry (IUPAC) nomenclature (see Section 8.2 for more details).

8. When the editing is completed, import the revised Cambridge Reference Sequence (rCRS) into the project. Highlight rCRS and the CONTIG(s) relevant to the sample (for example HV1 and HV2) in the project window and click on "assemble automatically". A new contig will be created which will consist of the sample's consensus sequence and the rCRS.

   Note: rCRS may be imported and assembled with the sample sequences in step 4 above. The contig is named to reflect the sample designation and the Examiner's symbols. Ensure base calls adhere to the sequence nomenclature set forth in section 8.2.

9. Differences from rCRS can be viewed by either of the following methods. Highlight rCRS, go to Sequence-->Compare To--> Consensus. Alternatively, highlight the contig and go to Contig--> Compare Consensus to Reference.

10. Save the file by exporting it as text. Open the "Just Saved" file to add the necessary items

to ensure that the file contains the following information: Laboratory Number, symbols, sample ID, date and sequence ranges. Print the file. The Sequencher sheets can now be used to compare and interpret differences among samples. Print out PROJECT VIEW. Note, using the letters "NA," the sequences which are not analyzable. Date and initial the PROJECT VIEW printout.

8.2 Sequence Nomenclature

1. Polymorphisms are noted on the Sequencher printouts and on the Case Summary Sheets. They are noted by stating the nucleotide position followed by the code for the polymorphic base (for example, 263G). Nucleotide base positions are designated according to the standard nomenclature set by the International Union of Physical and Applied Chemistry (IUPAC) and listed it the table below:

CODE	DESIGNATION
A	**Adenosine**
B	C,G, or T
C	**Cytosine**
D	A, G, or T
G	**Guanosine**
H	A, C, or T
K	G or T (Keto)
M	A or C (aMino)
N	aNy base
R	A or G (puRine)
S	G or C
T	**Thymine**
V	A, C, or G
W	A or T
Y	C or T (pYrimidine)

OTHER	DESIGNATION
-	Deletion
X.1(.2,...)	Insertion after position X

2. Profiles are characterized so that the least number of differences from rCRS are present. Insertions are listed to the right of a particular nucleotide position to minimize the number of differences from the rCRS. Insertions are described by first noting the site immediately 5' to the insertion followed by a point and a "1" (for the first insertion), a "2" (if there is a

second insertion), and so on, and then by the nucleotide that is inserted (ex. 315.1 C).

If there is more than one way to maintain the same number of differences with respect to rCRS, differences should be prioritized as follows: insertions/deletions, transitions, and, finally, transversions. Exceptions to this prioritization may occur based on historical nomenclature practices in the forensic mitochondrial DNA community (e.g. nucleotide positions 150 and 152).

3. When reporting length heteroplasmy, both minor and major peaks must be noted. Length heteroplasmy in HV1 most commonly arises when there is a substitution of a C for a T at position 16189. The reference type in HV1 is C_5TC_4. HV1 length heteroplasmy will not be recorded in casework samples. Rather, sequences will be truncated to fit the C_5TC_4 format. Length variants in HV2 are commonly observed in the number of C residues preceding a T residue at position 310. It is often possible to determine unambiguously the dominant length variant in this region, as well as some or all of the minor length variants, and the sequence is notated to reflect these variants. The reference type in HV2 is C_7TC_5. A 309.1C insertion in HV2 will be annotated C_8TC_5, and the insertions 309.1C, 315.1C will be annotated as C_8TC_6. Where a sequence does not have a T at 310 and length heteroplasmy is present, it is notated 310 C and no insertions or deletions relative to rCRS at 303-315 are noted. If a sequence does not have a T at 310 and length heteroplasmy is not present, then the guidance described in step 2 above can be used.

3. Typically, the range is defined as the span of double-stranded sequence obtained and denoted by the positions relative to the rCRS. In some instances, at the Examiner's discretion, single stranded range at the 5' or 3' end (and specifically noted on the case summary sheet as single-stranded) or surrounding the HV1 C-stretch region (if length heteroplasmy is present) may be included.

8.3 Sequence Comparisons

8.3.1 Evaluation of Control Sequences

The positive control run with the sample must type correctly in order to report the sequence for that sample.

After RB and NC sequence data are analyzed, a determination is made as to whether any analyzable sequence is present. If there is not, proceed to Section 8.3.2, "Comparison Among Samples." If there is, the sequence obtained from that sample must first be compared to any sequence obtained from the RB and NC associated with that sample before comparing it to another sample in the case. To determine whether any contamination seen in the RB or NC is the not the source of the DNA from the sample, the sequence of the sample and the corresponding RB or NC must differ by at least one base within each region (HV1a, HV1b, HV2a, HV2b, or HV1, HV2).

If the sequence of the RB or NC within a region is the same as that of the sample, the sample may be re-extracted, re-amplified, or the region not included in the reported sequence for that sample.

8.3.2 Comparison Among Samples

When comparing sequences obtained from samples, only the regions with a common range will be evaluated. For example, if a partial sequence (16024-16365 and 73-284) is obtained for a sample of questioned origin (Q), and a full sequence is obtained for the sample of known origin (K), the comparison will be conducted on positions 16024-16365 and 73-284. In addition, sequence before and after the defined HV1 and HV2 regions will also be used for comparison purposes, provided this range is common to both samples.

8.3.2.1 Comparisons Among Sequences with Length Heteroplasmy

Length heteroplasmy in HV1 most commonly arises when there is a substitution of a C for a T at position 16189. The reference type in HV1 is C_5TC_4. HV1 length heteroplasmy will not be recorded in casework samples. Rather, sequences will be truncated to fit the C_5TC_4 format.

The number of C nucleotides exhibited in samples with HV2 length heteroplasmy is highly variable and care must be exercised when making comparisons. In order for sequence concordance to be declared, a common length variant must be observed in the samples being compared. For instance, if a questioned sample has predominately 7 C residues preceding the T at position 310, but shows the presence of 8 Cs as well, it is concordant with samples containing any mixture of length variants of 7 or 8 Cs.

Differences in sequences due to the absence of a common length variant are not exclusionary. For instance, if a questioned sample shows only the presence of 7 Cs and a known sample shows 9 Cs, no common length variant is found between the two samples and a difference at position 309 is noted. This difference should be considered in the context of the entire comparison and the appropriate conclusion reported as described in Section 8.3.3. Length variants will not be used to compare reagent blank and negative control sequences against the sample sequence.

Where a sequence does not have a T at 310 and length heteroplasmy is present, it is notated 310 C and no insertions or deletions relative to rCRS at 303-315 are noted. If a sequence does not have a T at 310 and length heteroplasmy is not present, then the guidance described in Section 9.2 can be used.

8.3.3 Interpretation of Sequence Comparisons

The following interpretations are available for sequence comparisons:

Exclusion: If the samples differ at two or more positions, they can be excluded as coming from the same source. Cases which involve samples which are

excluded need not be confirmed by a second qualified Examiner.

Inconclusive: If the samples differ at a single position, with no evidence of heteroplasmy (including non-overlap of length variants), the comparison should be reported as inconclusive. Samples differing at a single nucleotide position *and* having no common length variant at positions 303-309 will also be reported as inconclusive. Because of the possibility of undetected heteroplasmy, additional samples may be analyzed when Q and K sequences differ by a single base. These samples may include blood, buccal swabs, and hair. Hair fragments from a known hair standard may be combined and processed as a single known sample. Cases which result in inconclusive sample comparisons may be confirmed by a second qualified Examiner.

Cannot Exclude: If samples have the same sequence, or demonstrate sequence concordance, they cannot be excluded as coming from the same source. Sequence concordance is defined as having a common base, or HV2 length variant, at each position. For example, if one sample has evidence of a C and a T at a given position, and the other has a C, they share the C in common at that position and are concordant. Similarly, overlap of length variants is considered sequence concordance. However, if one sample has evidence of a C and a T at a given position, and the other has a G, these sequences are not concordant. Sequences which result in a conclusion of "cannot exclude" are confirmed by a second qualified Examiner.

8.3.4 Sequence Confirmation

At a minimum, matching samples in cases which involve samples with concordant sequences, and those samples which will be uploaded into NDIS, must be confirmed by a second qualified Examiner. Confirmation involves independently assembling sequences for the NC, RB, and evidentiary samples from the analysis data. Separate Sequencher printouts are generated and initialed by each Examiner. Confirmed positive control printouts are named with the symbols of both Examiners, are marked "OK", and are initialed by both of the Examiners. Confirmed sequence range is defined as the shortest length of sequence obtained by two Examiners. For these cases, proceed to Section 8.4, "Weight Assessment." For other cases, proceed to Section 9, "Procedure for Report Writing."

8.4 Weight Assessment

8.4.1 Databases

The FBI Laboratory maintains a database consisting of human mtDNA nucleotide sequence data

from a variety of individuals of European, Asian, and African ancestry. The forensic database consists of profiles generated by forensic DNA typing laboratories and their contractors, and is maintained in collaboration with the Scientific Working Group on DNA Analysis and Methods (SWGDAM) (Miller and Budowle, 2001). The database is updated when appropriate, incorporating information from a variety of sources. Updates to the database are documented. Superseded versions of the database are archived.

8.4.2 Searching Profiles

CODIS + mito Popstats v.1.4, 5071 v.4 is used to search casework profiles in the forensic database. The range of the profile to be searched consists of the shortest range of sequence agreed upon by the Examiners reviewing the data. Database profiles that are up to 2 bases different from the casework profile are printed out and considered by the reporting Examiner. Ambiguous base positions in both database samples and casework profiles are searched as an N (meaning the search includes all four bases at that position) and do not have any exclusionary value when compared with other sequences. Deletions are searched as a "-". Those database samples whose range(s) are fully included within the casework profile range are used in the database search result. Presently, this includes the regions 16024-16365 (HV1) and 73-340 (HV2). The database search result provides the number of database profiles that match the casework profile, as well the number of profiles in the database that differ by up to five positions.

8.4.3 Special Considerations

The number of C residues in samples with HV1 length heteroplasmy (in or around nucleotide positions 16183-16194; see Section 8.2) are not considered for comparison purposes.
All sequence polymorphisms in the sample searched are entered; however, all length variants at nucleotide positions 16193 and 309 are ignored in database searches of relevant concordant sequences. Hence, length variability in this region will not add any additional rarity to a database profile search.

The Examiner confirms that profiles listed as the same are in fact the same as the casework profile being searched.

8.4.4 Upper Bound Frequency Estimate

A confidence interval can be calculated from the results of a database search in order to estimate the population frequency of a profile. An upper 95% confidence interval value (upper bound) may be presented as a percentage of individuals within each population who are estimated to be included as potential contributors of a questioned profile. The upper bound estimate is dependent on the size of the database, and these estimates are therefore likely to change as the database size changes.

The following formulae may be used to calculate the upper bound estimate:

In cases where the profile has been observed in a database:

$p + 1.96 [(p) (1-p)/N]^{1/2}$ (1)

$p = x/N$, where x is the number of times a profile has been observed in a population and N is the number of profiles in that population.

In cases where the profile has not been observed in a database:

$1-\alpha^{1/N}$ (2)

α is the confidence coefficient (0.05 for a 95% confidence interval), and N is the number of individuals in the population.

The results of these upper bound frequency estimate calculations for the three major population groups in the United States (African-American, Caucasian, and Hispanic) are included in the report. The calculations for other groups, whose total number in the database exceeds 100, may be included in the report when deemed appropriate by the Examiner.

9 Procedure for Report Writing

When a CODIS + mito Popstats search is conducted, the report will include a database table containing the search results of the three major population groups of the United States: African-American, Caucasian, and Hispanic. The table may also contain other applicable populations when deemed appropriate by the Examiner. In addition, the report will contain the search results of any other population where a matching type is found. An upper bound frequency estimate will typically be calculated only for population groups of 100 or more individuals.

The examples which follow are meant to represent typical case situations. Determination of the exact wording is made on a case-by-case basis.

9.1 Cases in which the DNA Sequences are Found to be the Same

Example 1

Mitochondrial DNA (mtDNA) sequences were obtained from the Q1 hair from the crime scene and the K1 blood sample identified as coming from JOHN DOE. The mtDNA sequences obtained from specimens Q1 and K1 are the same. Therefore, JOHN DOE cannot be excluded as the source of the Q1 hair.

Searching the mtDNA population database currently available to the FBI Laboratory (CODIS + mito Popstats v.1.4, 5071 v.4, containing 5071 individuals, searching positions 16024-16365 and 73-340), the mtDNA sequence obtained from specimens Q1 and K1 has been observed in the following major population groups:

Group	Number of Observations	Individuals in Group	Upper Bound Frequency Estimate
African-American	0	1148	0.26%
Caucasian	0	1814	0.17%
Hispanic	0	759	0.39%

The mtDNA sequencing results are detailed below. Results are listed as differences from the published revised Cambridge Reference Sequence (rCRS).

Specimen	**Q1** hair	**K1** JOHN DOE	**K2** JANE DOE
Range	np 15998-16389	np 15998-16389	np 15998-16388
HV1	16124 C 16223 T 16319 A	16124 C 16223 T 16319 A	16114 A 16129 A 16213 A 16223 T 16278 T 16355 T 16362 C
Range	np 49-380	np 49-407	np 49-407
HV2	73 G 150 T 152 C 263 G 315.1 C	73 G 150 T 152 C 263 G 315.1 C	73 G 150 T 152 C 182 T 195 C 198 T 204 C 263 G 315.1 C

No other mtDNA examinations were conducted.

Example 2

Mitochondrial DNA (mtDNA) sequences were obtained from the Q1 hair from the crime scene and the K1 blood sample identified as coming from JOHN DOE. The mtDNA sequences obtained from specimens Q1 and K1 are the same. Therefore, JOHN DOE cannot be excluded as the source of the Q1 hair.

Searching the mtDNA population database currently available to the FBI Laboratory (CODIS + mito Popstats v.1.4, 5071 v.4, containing 5071 individuals, searching positions 16024-16365 and 73-340), the mtDNA sequence obtained from specimens Q1 and K1 has been observed in the following major population groups:

Database	Number of Observations	Individuals in Database	Upper Bound Frequency Estimate
African-American	0	1148	0.26%
Caucasian	0	1814	0.17%
Hispanic	0	759	0.39%
China/Taiwan	2	356	1.34%

The mtDNA sequencing results are detailed below. Results are listed as differences from the published revised Cambridge Reference Sequence (rCRS).

[Insert mtDNA sequencing results table as shown in Example 1 here]

No other mtDNA examinations were conducted.

Example 3

Mitochondrial DNA (mtDNA) sequences were obtained from the Q1 hair from the crime scene and the K1 blood sample identified as coming from JOHN DOE. The mtDNA sequences obtained from specimens Q1 and K1 are the same. Therefore, JOHN DOE cannot be excluded as the source of the Q1 hair.

Searching the mtDNA population database currently available to the FBI Laboratory (CODIS + mito Popstats v.1.4, 5071 v.4, containing 5071 individuals, searching positions 16024-16365 and 73-340), the mtDNA sequence obtained from specimens Q1 and K1 has been observed in the following major population groups:

Database	Number of Observations	Individuals in Database	Upper Bound Frequency Estimate
African-American	0	1148	0.26%
Caucasian	0	1814	0.17%
Hispanic	0	759	0.39%
China/Taiwan	2	356	1.34%

In a search of population groups of limited size, this sequence was also seen 1 time in a population of 52 individuals from Thailand.

The mtDNA sequencing results are detailed below. Results are listed as differences from the published revised Cambridge Reference Sequence (rCRS).

[Insert mtDNA sequencing results table as shown in Example 1 here]

No other mtDNA examinations were conducted.

9.2 Cases in which the DNA Sequences are Found to be the Same, but Sequence was not Obtained from all Four Regions

Example

Mitochondrial DNA (mtDNA) sequences were obtained from the Q1 hair from the crime scene (nucleotide positions 16005-16236 and 49-408 only) and the K1 blood sample identified as coming from JOHN DOE. The mtDNA sequences obtained from specimens Q1 and K1 are the same. Therefore, JOHN DOE cannot be excluded as the source of the Q1 hair.

Searching the mtDNA population database currently available to the FBI Laboratory (CODIS + mito Popstats v.1.4, 5071 v.4, containing 5071 individuals, searching positions 16024-16236 and 73-340), the mtDNA sequence obtained from specimens Q1 and K1 has been observed in the following major population groups:

[Insert database search results table as shown in Example 1 here]

The mtDNA sequencing results are detailed below. Results are listed as differences from the published revised Cambridge Reference Sequence (rCRS).

[Insert mtDNA sequencing results table as shown in Example 1 here]

No other mtDNA examinations were conducted.

9.3 Cases in which the DNA Sequences are Found not to be in Concordance

Example:

Mitochondrial DNA (mtDNA) sequences were obtained from the Q1 hair from the crime scene and the K1 blood sample identified as coming from JOHN DOE. The mtDNA sequences obtained from specimens Q1 and K1 are different. Therefore, JOHN DOE can be excluded as the source of the Q1 hair.

The mtDNA sequencing results are detailed below. Results are listed as differences from the published revised Cambridge Reference Sequence (rCRS).

[Insert mtDNA sequencing results table as shown in Example 1 here]

No other mtDNA examinations were conducted.

9.4 Cases in which the DNA Sequences of the Questioned Sample and One Known Sample are Found to be the Same and the DNA Sequences of the Questioned Sample and a Different Known Sample are Found not to be in Concordance

Example

Mitochondrial DNA (mtDNA) sequences were obtained from the Q1 hair from the crime scene, the K1 blood sample identified as coming from JOHN DOE, and the K2 blood sample identified as coming from MICHAEL GRANT. The mtDNA sequences obtained from specimens Q1 and K1 are the same. Therefore, JOHN DOE cannot be excluded as the source of the Q1 hair.

Searching the mtDNA population database currently available to the FBI Laboratory (CODIS + mito Popstats v.1.4, 5071 v.4, containing 5071 individuals, searching positions 16024-16365 and 73-340), the mtDNA sequence obtained from specimens Q1 and K1 has been observed in the following major population groups:

[Insert database search results table as shown in Example 1 here]

The mtDNA sequence obtained from specimen K2 is different from the sequence obtained from Q1. Therefore, MICHAEL GRANT can be excluded as the source of the Q1 hair.

The mtDNA sequencing results are detailed below. Results are listed as differences from the published revised Cambridge Reference Sequence (rCRS).

[Insert mtDNA sequencing results table as shown in Example 1 here]

No other mtDNA examinations were conducted.

9.5 Cases in which the DNA Sequences in the Questioned and Known Samples are in Concordance and Sequence Heteroplasmy is Present

Example

Mitochondrial DNA (mtDNA) sequences were obtained from the Q1 hair from the crime scene and the K1 blood sample identified as coming from JOHN DOE. The mtDNA sequence obtained from specimen Q1 is the same as the mtDNA sequence obtained from specimen K1, with the exception of position 16069. At this position, the presence of a thymine (T) was observed in the Q1 hair. In specimen K1, evidence of both a thymine (T) and a cytosine (C) was characterized at position 16069.

Due to the closely related sequences obtained from specimens Q1 and K1, JOHN DOE cannot be excluded as the source of the Q1 hair.

Searching the mtDNA population database currently available to the FBI Laboratory (CODIS + mito Popstats v.1.4, 5071 v.4, containing 5071 individuals, searching positions 16024-16365 and 73-340), the mtDNA sequence obtained from specimens Q1 and K1, including all four nucleotides, A,C,G, and T, at position 16069, has been observed in the following major population groups:

[Insert database search results table as shown in Example 1 here]

The mtDNA sequencing results are detailed below. Results are listed as differences from the published revised Cambridge Reference Sequence (rCRS).

[Insert mtDNA sequencing results table as shown in Example 1 here]

No other mtDNA examinations were conducted.

9.6 Cases in which the DNA Sequences in the Questioned and Known Samples are in Concordance and Length Heteroplasmy is Present

Example

Mitochondrial DNA (mtDNA) sequences were obtained from the Q1 hair from the crime scene and the K1 blood sample identified as coming from JOHN DOE. The mtDNA sequences obtained from specimens K1 and Q1 are the same with the following exception at positions 303-310. At positions 303-310, specimen Q1 exhibited predominantly seven cytosine residues (with evidence

of eight cytosine residues as well) and specimen K1 exhibited predominantly eight cytosine residues (with evidence of seven cytosine residues as well).

Due to the closely related sequences obtained from specimens Q1 and K1, JOHN DOE cannot be excluded as the source of the Q1 hair.

Searching the mtDNA population database currently available to the FBI Laboratory (CODIS + mito Popstats v.1.4, 5071 v.4, containing 5071 individuals, searching positions 16024-16365 and 73-340), the mtDNA sequence obtained from specimens Q1 and K1 has been observed in the following major population groups:

[Insert database search results table as shown in Example 1 here]

The mtDNA sequencing results are detailed below. Results are listed as differences from the published revised Cambridge Reference Sequence (rCRS).

[Insert mtDNA sequencing results table as shown in Example 1 here]

No other mtDNA examinations were conducted.

9.7 Cases in which the DNA Sequences in the Questioned and Known Samples Differ by a Single Nucleotide, and No Evidence of a Common Nucleotide is Present in the Questioned and/or Known Sample at the Position of Difference

Example

Mitochondrial DNA (mtDNA) sequences were obtained from the Q1 hair, K1 blood sample, the K3 saliva sample, and the K5 head hair sample from JOHN DOE. The mtDNA sequence obtained from specimen Q1 is the same as those obtained from specimens K1, K3, and K5, with the exception of position 16069. At this position, the presence of a thymine (T) was observed in the Q1 hair. In specimens K1, K3 and K5, a cytosine (C) was characterized at position 16069.

Due to the one base pair difference observed between specimens Q1 and K1, K3, and K5, no conclusion could be reached as to whether JOHN DOE can be excluded as the source of the Q1 hair.

The mtDNA sequencing results are detailed below. Results are listed as differences from the published revised Cambridge Reference Sequence (rCRS).

[Insert mtDNA sequencing results table as shown in Example 1 here]

No other mtDNA examinations were conducted.

9.8 Cases in which the DNA Sequences in the Questioned and Known Samples Differ by a Single Nucleotide, and No Evidence of a Common Nucleotide is Present *and* No Evidence of a Common HVII Length Variant in the Questioned and/or Known Sample

Example

Mitochondrial DNA (mtDNA) sequences were obtained from the Q1 hair, K1 blood sample, the K3 saliva sample, and the K5 head hair sample from JOHN DOE. The mtDNA sequence obtained from specimen Q1 is the same as those obtained from specimens K1, K3, and K5, with the exception of positions 16069 and 303-310. At position 16069, the presence of a thymine (T) was observed in the Q1 hair. In specimens K1, K3 and K5, a cytosine (C) was characterized at position 16069. At positions 303-310, specimen Q1 exhibited seven cytosine residues and specimens K1, K3, and K5 exhibited nine cytosine residues.

Due to the one base pair difference and the lack of a common length variant observed between specimens Q1 and K1, K3, and K5, no conclusion could be reached as to whether JOHN DOE can be excluded as the source of the Q1 hair.

The mtDNA sequencing results are detailed below. Results are listed as differences from the published revised Cambridge Reference Sequence (rCRS).

[Insert mtDNA sequencing results table as shown in Example 1 here]

No other mtDNA examinations were conducted.

9.9 Specimens where there is an Apparent Mixture of More than One mtDNA Sequence

Example

The mitochondrial DNA (mtDNA) sequence obtained from specimen Q1 indicates the presence of a mixture of mtDNA from more than one individual. Because mixtures of mtDNA are not interpretable, no conclusions can be reached.

9.10 Cases in which Two Questioned Samples are in Concordance but there are no Known Samples available for Comparison or they do not Match the Known Samples Examined

Example

Mitochondrial DNA (mtDNA) sequences were obtained from the Q1 hair from the crime scene and the Q2 hair from the vacuum cleaner. The mtDNA sequences obtained from specimens Q1 and Q2 are the same. Therefore, Q1 and Q2 cannot be excluded as arising from a common source.

[No database search is run in this situation.]

The mtDNA sequencing results are detailed below. Results are listed as differences from the published revised Cambridge Reference Sequence (rCRS).

[Insert mtDNA sequencing results table as shown in Example 1 here]

No other mtDNA examinations were conducted.

9.11 Specimens where No Sequence was Obtained

Example

There was insufficient mitochondrial DNA (mtDNA) present for a DNA sequence to be obtained from the Q1 hair or the Q2 hair.

No other mtDNA examinations were conducted.

9.12 Cases in which No mtDNA Examination was Conducted because No Microscopic Associations were Made

Example

Because no microscopic associations between the questioned hairs and the known hair standards were made in this case, no mitochondrial DNA examinations were conducted.

9.13 Cases in which No mtDNA Examination was Conducted because Remains were Determined to be of Non-Human Origin

Example

The Q10 bone was examined by Dr. Jane Smith, Curator, Department of Anthropology, Smithsonian Institution, Washington, DC. Dr. Smith's report is enclosed herewith. Because the bone is not of human origin, no mitochondrial DNA examinations were conducted.

9.14 Cases in which the mtDNA is Not of the Requisite Quality:

The mitochondrial DNA (mtDNA) obtained from the Q1 hair was not of the requisite quality for an mtDNA sequence to be obtained.

9.15 National Missing Persons DNA Database (NMPDD) Cases

A mitochondrial DNA (mtDNA) sequence was obtained from the K1 buccal swab from MARY DOE, identified as the mother of JANE DOE (NCIC No. MXXXXXXXXXX). The mtDNA sequence from K1 will be entered into the National Missing Person DNA Database.

The mtDNA sequencing results will not typically be provided in NMPDD cases.

*The **Remarks** section of the report will include statements similar to those below:*

Mitochondrial DNA profiles from unidentified human remains that are uploaded to the National DNA Index System (NDIS) require a complete mtDNA profile (nucleotide positions 16024-16365 and 73-340). If uploading to NDIS is required, additional skeletal material is needed. Please submit more skeletal material such as a femur, tibia or other whole bone sample if available.

A portion of item Q2 and item Q1 will be retained for archival purposes for possible future testing. The remainder of the submitted items will be returned under separate cover along with the processed DNA generated from the samples. The processed DNA will be found in a package marked PROCESSED DNA SAMPLES: SHOULD BE REFRIGERATED/FROZEN. It is recommended that these samples be stored in a refrigerator/freezer and isolated from evidence that has not been examined.

The results of the nuclear DNA examination will be the subject of a separate report.

10 Equipment/Materials/Reagents

10.1 Reagents and Materials

The following are general instructions applicable to the preparation of all reagents used in the mtDNA protocol:

Use graduated cylinders or pipettes closest in capacity to the volume being measured for preparing liquid reagents.

Sterile, distilled deionized water (ddH_2O) must be used in preparing reagents. Formulations may be scaled up or down as necessary to meet laboratory needs.

Store all reagents in sterile containers. Label all reagents with name of reagent, lot number, date received or prepared, initials of preparer, QC date (if applicable), initials of person performing QC, the date the reagent is first used in the laboratory (start date) and the initials of the person first using it, expiration date (1 year from QC date if not otherwise specified), and the storage

conditions.

The reagents below are listed in alphabetical order. Other sources for reagents may be used. If other sources are used, molecular biology grade reagents are used where appropriate and available.

AmpliTaq Gold® DNA POLYMERASE

Applied Biosystems N808-0244, 5U/µL. Store at -20°C.

ARRAY FERRULE KNOB

Applied Biosystems 628-3730

ARRAY FERRULE SLEEVES

Applied Biosystems 628-0165

BLEACH SOLUTION

10% household bleach solution, 500 mL. Bring 50 mL household bleach to 500 mL with tap or house deionized water. Make fresh each day of use. Store at room temperature.

Alternatively, the Fresh Mix system with bleach cartridges may be used (Market Lab WL 0999 for starter kit, Market Lab WL 102 for bleach cartridge refills, and Market Lab WL 1060 for Fresh Mix bleach bottles). Store at room temperature.

BSA (1.6 mg/mL Bovine Serum Albumin)

Add 64 mg bovine serum albumin (e.g. Sigma A2153) to sterile ddH_2O (less than 40 mL). Gently stir until the BSA is dissolved. Adjust final volume to 40 mL with sterile ddH_2O to achieve a concentration of 1.6 mg/mL. Aliquot 60 µL portions and store at -20°C.

10X BUFFER (GeneAmp 10X PCR Buffer)

GeneAmp 10X Buffer (Applied Biosystems N808-0244) contains: 100 mM Tris-HCl, pH 8.3, 500 mM KCl, 15 mM $MgCl_2$, and 0.01% (w/v) gelatin. Store at -20°C.

CAPILLARIES (for ABI Prism 3130 Genetic Analyzers)

36 cm array for 3130, Applied Biosystems 4315931

CENTRI-SEP SPIN COLUMNS

Princeton Separations, Inc. CS-901

CENTRI-SEP PLATES

Princeton Separations, Inc. CS-961

CENTRI-STRIPS 8

Princeton Separations, Inc. CS-912

CUTTING DISCS

Economy cutting disks, #2 Medium, Henry Schein Dental 100-6804

CYLINDERS

Spex 6751 for the 6750 Freezer Mill; Spex 6804 for the 6800 Freezer Mill

DNA 1000 SERIES II LABCHIP KIT (25 microchips and reagents)

Agilent Technologies 5065-4449

DNA 7500 SERIES II LABCHIP KIT (25 microchips and reagents)

Agilent Technologies 5064-8230

DNA SEQUENCING KITS

dRhodamine Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems 403042) or 1000 reaction pack (403043). Prior to use, allow the kit to thaw to room temperature and briefly centrifuge. Store at -20^{o}C.

BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems 4337450). Prior to use, allow the kit to thaw to room temperature and briefly centrifuge. Store at -20^{o}C.

DEOXYRIBONUCLEOSIDE TRIPHOSPHATES (dNTPs) MIX

GeneAmp dNTP (Applied Biosystems N808-0260) mix is at 10 mM concentration, with each dNTP (dATP, dCTP, dGTP, and dTTP) at 2.5 mM concentration. Store at -20^{o}C.

DNA-OFF

Daigger LT12982 or Pure Biotech D0500. Store at room temperature.

DREMEL Motor Tool

Model 300 Series, Dremel, Racine, Wisconsin

DTT (Dithiothreitol, $C_4H_{10}O_2S_2$)

Sigma-Aldrich D-9779-10G or VWR EM-3860. Molecular biology grade. Store at -20^{o}C.

EDTA (Ethylenediaminetetraacetic Acid Disodium Salt $C_{10}H_{14}N_2O_2NA\cdot 2H_2O$, 0.5 M, pH 8.0)

Sigma-Aldrich E7889 0.5 M solution, pH 8.0. Store at room temperature.

EDTA (for calcified tissue extraction)

Sigma-Aldrich E-7889 0.5 M solution, pH 8.0. Store at room temperature.

EMERY DISKS

Fine, 7/8" (Moore's Discs with brass center), Henry Schein Dental 294-4369

ETHANOL (EtOH) ABSOLUTE

200 Proof, Molecular Biology grade (Sigma-Aldrich E7023 -500 mL). Store at room temperature in flammable liquids cabinet.

EXO SAP-IT

United States Biochemical 78202 or GE Healthcare US78202.

EXTRACTOR

Spex 6754

EXTRACTION TUBES

Sarstedt 72.695.200 2.0 mL Biosphere tubes. Certified DNase and RNase free.

EXTRACTION BASKETS

Costar 9301 or Fisher NC921-71-72

EZ-Window

Agilent, 590-3001

FACE MASKS

Clean room face masks, Kimberly-Clark GPM Face Mask, Kimberly-Clark 62477 or VWR 0843-203

FORMAMIDE (HI-DI, for ABI PRISM 3130 Genetic Analyzers)

Applied Biosystems 4311320

CAUTION: Formamide is an embryotoxin. This substance should be used in a chemical hood. Precautions should be taken to avoid skin contact.

10X GENETIC ANALYZER BUFFER

Applied Biosystems, Inc. 402824

HYDROCHLORIC ACID (HCl, concentrated)

Fisher A-144, Reagent ACS grade, 36.5-38.0% or Sigma-Aldrich 258148-25 mL, ACS reagent 37%.

METHANOL (CH_3OH, HPLC GRADE)

Fisher A452-4 HPLC grade. Store at room temperature in a flammable liquids cabinet.

MICROAMP RETAINER

Applied Biosystems N801-0530

MICROCENTRIFUGE TUBES

Sarstedt 72.735 0.5 mL Biosphere tubes. Certified DNase and RNase free.

MICROCENTRUFUGE TUBES

Sarstedt 72.690.200 1.5 mL Biosphere tubes. Certified DNase and RNase free.

MICRO TISSUE GRINDER

Kontes, Tissue Grinder, Micro 885470-0000 (Fisher K885470-0000).

MICROCON 100

Filters Amicon/Millipore 42413. Extra collection tubes (100 pk) Amicon/Millipore 1065601.

PCIA (Phenol / Chloroform / Isoamyl alcohol (25:24:1; v/v))

Sigma P3803

CAUTION: PCIA is an irritant and is toxic. Its use must be confined to a fume hood. Gloves and a mask should be worn.

PLATE SEPTA (96 well)

For 3130 Genetic Analyzer, Applied Biosystems 4319533.

POP-6

Applied Biosystems 4363783

POSITIVE CONTROL DNA

HL60 Cell line control DNA is purchased from ATCC (45501). Dilute to 10 pg/µL using ddH_2O and store at -20°C or colder.

PRIMERS (Oligonucleotide DNA Primers)

Primers are custom synthesized by Invitrogen Life Technologies or other suitable vender that can supply primers of requisite quality. Synthesized primers are HPLC-purified and quantified. Lyophilized primers are stored at -20°C. Each primer is reconstituted from the lyophilized form using ddH_2O to achieve a final concentration of 100 µM (100 nanomoles/mL). Storage stocks should consist of small aliquots stored at -20°C.

1-PROPANOL

1-Propanol, Fisher A414-500.

PROTEINASE K (20 mg/mL),

AMRESCO, Inc. E195-25 mL. Store 5 µL aliquots at 4°C. Discard any unused portions of tubes at each use.

QIAamp DNA Mini Kit

Mini Kit (AW1 and AW2 wash buffers) QIAGEN 51306. AL buffer kit (set of 4) QIAGEN 19075. Store at room temperature.

REACTION PLATES

Applied Biosystems N801-0560

REACTION CAPS (MicroAmp Caps)

Applied Biosystems N801-0535

REACTION TUBES (MicroAmp Reaction Tubes)

Applied Biosystems N801-0533

REACTION TUBES (Eppendorf)

Fisher E0030124 286

RESERVOIR (BUFFER, WATER, WASTE)

For 3130 Genetic Analyzer (96-well), Applied Biosystems 628-0163

RESERVOIR SEPTA

For 3130 Genetic Analyzer (96-well), Applied Biosystems 4315932

SDS 20% ((w/v) Sodium Dodecyl Sulfate)

Sigma-Aldritch Electrophoresis Grade solution 05030. Store at room temperature.

SEB (STAIN EXTRACTION BUFFER, 10 mM TRIS - 100 mM NaCl - 39 mM DTT - 10 mM EDTA - 2% SDS)

To make a 1 L stock solution, dissolve 5.84 g sodium chloride (NaCl, e.g. Sigma S-3014 molecular biology grade) in 500 mL sterile ddH_2O. Add 10 mL 1 M Tris-HCl, 20 mL 0.5 M EDTA, and 100 mL 20% SDS. Titrate to pH 8.0 with HCl. Bring final volume to 1 L with ddH_2O. Store at room temperature.

Supplement with DTT (30 mg DTT per 5 mL SEB solution) and expose to UV light in a crosslinker for a minimum of 15 minutes before use. Make fresh for each use.

SODIUM CHLORIDE (NaCl)

Sigma-Aldrich S-3014 Molecular Biology Grade. Store at room temperature.

SULFURIC ACID (4 N H_2SO_4)

Fisher SA 818-500. Store at room temperature in an acid cabinet.

TERGAZYME

VWR 21837-118 (Alconox Inc.) or Fisher 04-322-11A

TRIS-HCL, 1 M, pH 8.0

Sigma-Aldritch T-2694 Molecular Biology Grade. Store at room temperature.

WATER

High Purity Water, CAP and NCCLS, VWR 72060-094. This water is used for all procedures unless otherwise indicated. It is subjected to 15 minutes of UV light treatment where indicated.

WATER, NUCLEASE FREE

For preparation of critical reagents such as positive control DNA and primers. Sigma-Aldrich W3513. Expose to UV light in a crosslinker for a minimum of 15 minutes prior to use.

XYLENE SUBSTITUTE

Shandon Histosolve Xylene Substitute, Thermo Electron Corporation 9990505. Store at room temperature in a flammable liquids cabinet.

CAUTION: Xylene Substitute is flammable and has harmful vapors. Its use must be confined to a fume hood. Gloves and a mask should be worn.

10.2 Equipment

Use specified equipment. If parenthetical statement says "e.g.," then equivalent equipment can be used.

ABI Prism 3130 Genetic Analyzers
Agilent 2100 Bioanalyzer
Agilent Priming Station
Autoclave
Freezers (-20^{o}C, -80^{o}C)
Freezer Mill (SPEX Centriprep 6700 or 6750)
Hoods - Laminar Flow w/ UV light source (e.g. Labconco Purifier Class II Biosafety Cabinet)
Hood - Ductless Fume w/ HEPA filter (e.g. AirClean 600 Work Station)
Hood - Ductless w/ Particulate Filter (e.g. Mystaire 2450)
Hot Plate (e.g. Corning Stirrer/Hot Plate)
Incubator (e.g. Labnet Digital Incubator 311D, Midwest Scientific)
Microcentrifuges (fixed angle, capable of at least 13000 X g and of holding 2 mL tubes)
Nanopure Water System (e.g. Nanopure Infinity)
Nutator (117 V, Clay Adams, from Fisher)
Orbital Shaker (e.g. Labnet Shaker 31)
Pipette, Repeater (e.g. Brinkmann Eppendorf Repeater Pipette)
Pipettors (various sizes, to cover 1 µL to 1000 µL)
Refrigerators
Rocker (e.g. Hoefer Red Rocker)
Stereomicroscope (Bausch and Lomb Stereozoom 7 or Leica)
Thermal Cycler (Applied Biosystems, Inc. GeneAmp System 9700)
Thermometers, to cover -20^{o}C, 4^{o}C, 50^{o}C, 56^{o}C, 95^{o}C (NIST-traceable)
Touch Mixers/Vortexes (e.g. Fisher Vortex Genie 2)
Ultrasonic Water Bath
UV Crosslinker (e.g. Krackeler Scientific Spectrolinker XL-1500)
Vortemp or Thermomixer Incubator
Vortex Mixer (e.g. Labnet VX-100 Vortex Mixer or Scientific Industries Vortex-Genie 2 Mixer)
Vacuum Sample Evaporator Centrifuge (e.g. Savant Automatic Environmental Speedvac System)

11 Suppliers

A list of the companies that supply the appropriate quality of reagents, supplies, and equipment follows.

Agilent Technologies 1-800-227-9770
13017 Wisteria Drive, #600
Germantown MD 20874
www.agilent.com

Airclean Systems 1-800-849-0472
3348 Lake Woodard Drive
Raleigh NC 27604

Aldrich 1-800-558-9160
1001 W. St. Paul Ave.
Milwaukee WI 53233
www.sigma-aldrich.com

Amersham Biosciences 1-800-526-3593
800 Centennial Ave.
P.O. Box 1327
Piscataway NJ 08855-1327

Amicon® (Millipore) 1-800-426-4266
17 Cherry Hill Dr.
Danvis MA 01923
www.millipore.com

Amresco® 1-800-829-2805
30175 Solon Industrial Pkwy.
P.O. Box 39098
Solon OH 44139
www.amresco.com

Applied Biosystems 1-800-327-3002
850 Lincoln Center
Foster City CA 94404
www.appliedbiosystems.com

ATCC 703-365-2700
10801 University Blvd.
Manassas VA 20110-2209
www.atcc.org

Bellco Glass, Incorporated
349 Edrudo Road
Vineland NJ 08360
www.bellcoglass.com

Daigger 1-800-621-7193
620 Lakeview Parkway
Vernon Hills, IL 60061
www.daigger.com

Dremel® 1-800-437-3635
P.O. Box 1468
Racine WI 53401
www.dremel.com

Fisher 1-800-766-7000
3970 Johns Creek Ct.
Suite 500
Suwanee GA 30024
www.fishersci.com

GE Healthcare 1-800-526-3593
800 Centennial Ave.
Piscataway, NJ 08855
www.gehealthcare.com

Government Scientific Source, Inc. (GSS) 1-800-248-8030
8460 Tyco Rd.
Vienna, VA 22182
www.govsci.com

GIBCO/BRL 1-800-828-6686
Life Technologies®
3175 Staley Rd.
Grand Island NY 14072
www.lifetech.com

Invitrogen Corporation 1-800-955-6288
12088 Collections Center Dr.
Chicago, IL 60693
www.invitrogen.com

ISC BioExpress 1-800-999-2901
420 North Kays Dr.
Kaysville, UT 84037
www.bioexpress.com

Kontes 1-800-253-7150
Scientific Glassware/Instruments
1022 Spruce St.
Vineland NJ 08360
www.kontes.com

Krackeler Scientific, Inc. 1-800-334-7725
P.O. Box 1849
Albany, NY 12201
www.krackeler.com

Labconco 1-816-333-8811
8811 Prospect
Kansas City MO 64132

MarketLab Inc.
P.O. Box 888374
Kentwood MI 49588-8374
616-656-2484
www.marketlabinc.com

Marsh/Abgene 1-800-445-2812
565 Blossom Rd.
Rochester, NY 14610
www.marshbio.com

Midwest Scientific 1-800-227-9997
280 Vance Road
Valley Park MO 63088
www.midsci.com

Millipore Corporation 1-800-MILLIPORE
186 Middlesex Turnpike
Burlington, MA 01803

Misonix 1-800-645-9846
1938 New Highway
Farmingdale NY 11735
PGC Scientifics Corporation 1-800-424-3300
7311 Governors Way
Frederick, MD 21704
www.pgcsci.com

Phenix Research Products 1-800-767-0665
1463 Sand Hill Rd.
Candler, NC 28715
www.phenixresearch.com

Princeton Separations 1-732-431-3338
P.O. Box 300
Adelphia NJ 07710
www.prinsep.com

Pure Biotech 1-866-252-7771
752A Lincoln Blvd.
Middlesex, NJ 08846
www.purebiotechllc.com

QIAGEN, Inc. 1-800-426-8157
28159 Avenue Stanford
Valencia CA 91355-1106

Rainin 1-800-472-4646
7500 Edgewater Drive
Oakland CA 94621

Roche Molecular (Boehringer Mannheim) 1-800-262-1640
9115 Hague Road
P.O. Box 50414
Indianapolis IN 46250-0414
http://biochem.roche.com

Sarstedt, Inc. 1-800–257-5101
P.O. Box 468
Newton NC 28658-0468
www.sarstedt.com

SIGMA 1-800-325-3010
P.O. Box 14508
St Louis MO 63178
www.sigma-aldrich.com

Spex CertiPrep, Inc. 1-800-522-7739
203 Norcross
Metuchen NJ 08840
www.spexcsp.com

Henry Schein 1-800-851-0400
5 Harbor Park Drive
Port Washington NY 11050
www.henryschein.com

Thermo Electron Corporation
171 Industry Drive
Pittsburgh PA 15275-1034
800-547-7429
www.thermo.com

VWR/Scientific Products 1-800-932-5000
P.O. Box 626
Bridgeport NJ 088014
www.vwrsp.com

Whatman BioScience 1-866-787-3362
200 Wells Avenue
Newton MA 02459-3347
www.whatman.com

12 Sampling

Not applicable.

13 Calibration

Pipettors and the ABI Prism 3130 Genetic Analyzer are subject to calibration. This is addressed in Section 2, Quality Assurance (Section 2.3.7 for pipettes and 2.3.9 for the ABI Prism 3130 Genetic Analyzer).

14 Calculations

Calculations are performed to determine the concentration of amplified mitochondrial DNA. These calculations, described in Section 5.1.3.1 of Post-Amplification Quantification by Capillary Electrophoresis, are used to determine the volume of amplified DNA subjected to cycle sequencing. An Excel spreadsheet is used to automate these calculations and is part of the case notes.

A 95% confidence interval is calculated by the CODIS + mito Popstats v.1.4, 5071 v.4 software in order to determine an upper bound frequency estimate of individuals within each population who are estimated to be included as potential contributors of a questioned profile. This is described in Section 8.4.4.

15 Uncertainty of Measurement

Not applicable.

16 Limitations

Mitochondrial DNA analysis is subject to limitations defined in the extensive validation studies conducted by FBI scientists prior to the implementation of the procedure. Publications describing the validation are listed in the References section. These limitations concern the evaluation and interpretation of control samples as described in Section 8.3 of Sequence Analysis, Comparison, and Interpretation (evaluation) and Section 2.5 of Quality Assurance (interpretation).
In addition, validation studies show that mixtures of mtDNA from more than one individual are not interpretable, and are reported as such.

17 Safety

Hazards associated with the procedure are stated throughout the procedure where applicable. The specific hazards noted are in the following sections:

Section 2.3.4 UV Crosslinker

Section 3.1, Procedure for DNA Extraction from Hair

Section 3.2, Procedure for DNA Extraction from Body Fluids and Tissues

Section 3.3.6, Procedure for DNA Extraction from Calcified Tissue

Section 5.1 Procedure for using the Agilent Technologies 2100 Bioanalyzer

Section 7, Procedure for Sequencing Using the ABI Prism 3130 Genetic Analyzer

Section 10, Equipment/Materials/Reagents

Section 21.2 Appendix B – Procedure for the Preparation of Dried Bloodstains

18 References

Anderson, S. *et al.* Sequence and organization of the mitochondrial genome, *Nature* (1981) 290:457-465.

Anderson, T. *et al.* A validation study for the extraction and analysis of DNA from human nail material and its application to forensic casework, *Journal of Forensic Sciences* (1999) 44:1053-1056.

Andrews, R. M. *et al.* Reanalysis and revision of the Cambridge reference sequence for human mitochondrial DNA, *Nature Genetics* (1999) 23:147.

Budowle, B. *et al.* Mitochondrial DNA regions HVI and HVII population data, *Forensic Science International* (1999) 103:23-35.

Budowle B. *et al.* Forensics and mitochondrial DNA: applications, debates, and foundations, *Annual Review of Genomics and Human Genetics* (2003) 4:119-141.

Butler, J. M. *et al.* Quantitation of PCR products by capillary electrophoresis using laser fluorescence, *Journal of Chromatography A* (1994) 658:271-280.

Koop, B. F. *et al.* Sequence length and error analysis of Sequenase and automated *Taq* cycle sequencing methods, *BioTechniques* (1993) 14:442-447.

Levin, B. *et al.* Comparison of the complete mtDNA genome sequences of human cell lines -

HL60 and GM10742A - from individuals with pro-myelocytic leukemia and leber hereditary optic neuropathy, respectively, and the inclusion of HL60 in the NIST human mitochondrial DNA standard reference material - SRM 2392-I, *Mitochondrion* (2003) 2:387-400.

McCord, B. R. *et al.* Capillary electrophoresis of PCR-amplified DNA using fluorescence detection with an intercalating dye, *Journal of Chromatography A* (1993) 652:75-82.

McCord, B. R. *et al.* High resolution capillary electrophoresis of forensic DNA using a non-gel sieving buffer, *Journal of Chromatography A* (1993) 16:1963-1981.

Miller, K. W. P. *et al.* A compendium of human mitochondrial DNA control region: development of an international standard forensic database, *Croatian Medical Journal* (2001) 42:315-327.

Monson, K. L. *et al.* The mtDNA population database: in integrated software and database resource for forensic comparison, *Forensic Science Communications* (2002), volume 4, number 2.

Parker, L. T. *et al.* Amplitaq DNA polymerase, FS dye-terminator sequencing: analysis of peak height patterns, *BioTechniques* (1996) 21:694-699.

Rosenblum, B. B. *et al.* New dye-labeled terminators for improved DNA sequencing patterns, *Nucleic Acids Research* (1997) 25:4500-4504.

Saiki, R. K. *et al.* Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase, *Science* (1988) 239:487-491.

Sanger, F. *et al.* DNA sequencing with chain-terminating inhibitors, *Proceedings of the National Academy of Sciences USA,* (1977) 74:5463-5467.

Stewart, J. E. B. *et al.* Length variation patterns in the human mitochondrial DNA control region, *Journal of Forensic Sciences* (2001) 46:95-102.

Stewart, J. E. B. *et al.* Evaluation of a multicapillary electrophoresis instrument for mitochondrial DNA typing, *Journal of Forensic Sciences* (2003) 48:571-580.

SWGDAM, Guidelines for mitochondrial DNA (mtDNA) nucleotide sequence interpretation, *Forensic Science Communications* (2003), volume 5, number 2.

Wilson, M. R. *et al.* Validation of mitochondrial DNA sequencing for forensic casework analysis, *International Journal of Legal Medicine* (1995) 108:68-74.

Wilson, M. R. *et al.* Extraction, PCR amplification, and sequencing of mitochondrial DNA from human hair shafts, *BioTechniques* (1995) 18(4):662-669.

Wilson, M. R. et al. Further discussion of the consistent treatment of length variants in the human mitochondrial DNA control region, *Forensic Science Communications* (2002), volume 4, number 4.

Zakeri, H. *et al.* Peak height pattern in dichloro-rhodamine and energy transfer dye terminator sequencing, *BioTechniques* (1998) 25:406-414.

19 Standards and Controls

19.1 Standards

NIST Standard Reference Material 2392-I (HL60) is the standard used for mtDNA analysis. Its use is described in Section 2.4.5 of the DNA Analysis Unit II Quality Assurance Manual.

19.2 Controls

The controls used throughout mtDNA analysis are the reagent blank (RB), negative control (NC), and positive control (HL60). The preparation of the RB is described in each extraction procedure (3.1.2 for Hair Extraction, 3.2 for Body Fluid/Tissue Extraction, and 3.3.3 for Calcified Tissue Extraction). The preparation of the NC and positive control is described in Sections 4.1.1, 4.1.2, and 4.1.3 DNA Amplification. The interpretation of control samples is described in Section 2.5 of Quality Assurance, and the evaluation of control samples is described in Section 8.3.1 of Sequence Analysis, Comparison, and Interpretation.

Additionally, there are controls used for post-amplification DNA quantification. These are the DNA 1000 markers for the Agilent DNA 1000 Series II LabChip Kit (Section 5.1.1.3 of Post-Amplification Quantification by Capillary Electrophoresis) and the DNA 7500 markers for the Agilent DNA 7500 Series II LabChip Kit (Section 5.1.2.3 of Post-Amplification Quantification by Capillary Electrophoresis). Further discussion of the use of these markers is in Section 5.1.3.1 of Post-Amplification Quantification by Capillary Electrophoresis.

20 Retention and Uploading of CODIS Samples

Mitochondrial DNA profiles that are to be uploaded into the CODIS system are recorded on the CODIS Case Evaluation Form. The form captures the mtDNA profile, the Specimen Identification number, the examiner of record and the sequence confirming examiner, the FBI Laboratory number, the FBI Case File number, the disposition (sample type), NCIC number and ViCAP number, if available. The form is completed, technically and administratively reviewed. The form is copied onto red paper. The original and red copy of the CODIS Case Evaluation Form, a copy of the incoming communication and paperwork submitted with the case (NCIC reports,

Anthropology reports, Dental reports, Law Enforcement reports), and a copy of the report are submitted to the NMPDD Program Manager for all cases with samples to be uploaded.

20.1 Specimen Identification Number

A standardized format for entering NMPDD mtDNA profile information into CODIS + mito has been devised. The specimen identification number will consist of the two letter symbol designation for the DNAU II Examiner of record followed by a backslash (/), the DNAU I Examiner of record for the case, the FBI Laboratory number and then the Questioned or Known item number.

Example 1

Specimen Number: DNAU II Examiner Symbol + / + DNAU I Examiner Symbol + Lab Number + Q/K Number.

Example 1	
The K1 reference sample from a Mother of a Missing Person submitted under FBI Laboratory number 040506001 PO KM (KM is the DNAU II examiner and KJ is the DNAU I examiner).	KM/KJ040506001K1

Example 2

Specimen Category: Unidentified Human Remains (Unidentified Person) Q3

Specimen Number: Examiner Symbol + Lab Number + Q/K Number

Example 2	
The Q3 Unidentified Human Remains submitted under FBI Laboratory number 040109001 KM.	KM/KJ040109001Q3

20.2 Data Review Requirements Prior to CODIS Entry and Uploading

The genetic analysis data for all mtDNA profiles generated in DNAU II must undergo primary analysis by the qualified DNAU II Examiner of record for the case and a confirmation by a second qualified DNAU II Examiner. Interpretation and Technical review of the data must be performed in accordance with this protocol.

20.3 NDIS Acceptable mtDNA Sequence Range

The acceptable sequence range for uploading a sample to NDIS includes positions 16024 to 16365 and 73 to 340 (HV1 and HV2). The sample profile range can exceed this range and still be acceptable for uploading to NDIS. Samples with mtDNA profiles that are less than the NDIS acceptable range will be searched by keyboard searches.

20.4 Uploading to SDIS or NDIS

CODIS Case Evaluation forms will be forwarded to the DNAU I NMPDD Program Manager or his/her designee for uploading into SDIS and later to NDIS.

20.5 Sample Retention

1. For each sample that is uploaded into the NMPDD, a portion (if available) will be retained and archived for possible re-analysis.

2. The archived samples will be retained in the NMPDD Repository -20^{o}C freezer. The archived sample will have the FBI Laboratory number, the FBI case file number and the Q or K designation on the outside of the packaging.

3. If available, a bone cutting or tissue sample from unidentified human remains of approximately 30 mm X 30 mm will be retained for possible retesting. Up to 4 buccal swabs will be retained from each biological relative of a missing person. Both sample cards (if available) from the blood cell collection kits will be retained.

4. The archived sample will be retained until an administrative removal or expungement request is exercised. The archived sample will then be returned to the contributor.

20.6 Reports

1. The FBI Laboratory report will summarize which specimens were sequenced and uploaded into the NMPDD.

2. The FBI Laboratory report will list the mtDNA sequence.

3. The FBI Laboratory report will explain that a portion of the sample is to be retained for archival purposes in the Remarks section.

4. If the original evidence, item, or extract generated in DNAU II from an item is to be typed by DNA Analysis Unit I for a nuclear DNA profile, this will be reflected in the Remarks section of the report.

5. If a partial profile is obtained for a specimen, the Remarks section of the report will state that the sequence cannot be uploaded into NDIS and that more skeletal material is needed if uploading into NDIS is desired.

21 Appendices

21.1 Appendix A - Positive Control Sequences

HL60 DNA is used as a positive control. The whole control region (WCR) of the mtDNA from this cell line has been amplified and sequenced by the FBI Laboratory. The HL60 mtDNA WCR sequence, expressed as differences from the rCRS, is provided in the table below. The mtDNA HV1 and HV2 sequences for the cell lines K562 and 9947A are provided for information.

Cell Line	HL60 (Whole Control Region)	9947A	K562
HV1	16069 T 16193 T 16278 T 16362 C	16311 C	16126 C 16294 T 16296 T 16324 C
HV2	73 G 150 T 152 C 263 G 295 T 315.1 C 489 C (outside of HV2)	93 G 195 C 214 G 263 G 309.1 C 309.2 C 315.1 C	73 G 263 G 310-313 D

21.2 Appendix B-Procedure For the Preparation of Dried Bloodstains

When liquid blood exemplars are submitted to the Laboratory, blood stains may be made from the liquid blood samples if desired using the following procedure.

Special Precautions: All biological materials must be considered pathogenic, and liquid blood specimens are especially hazardous. Liquid blood tubes must be handled only when wearing gloves. When opening liquid blood tubes, one must be wearing eye protection and mouth protection in addition to gloves. The use of double gloves is recommended when handling known AIDS or Hepatitis-infected materials. All operations with liquid blood samples, including drying, should be carried out in a dedicated laminar flow hood. Liquid

blood tubes should be stored packaged in mailing tubes designed for that purpose.

21.2.1 From Coagulated Blood

1. Label an FTA Blood Card with, at a minimum, the Laboratory number and sample identification (K) number.

2. Place a disposable leak-resistant mat beneath the Blood Card.

3. Remove the tube closure and place it on a disposable surface.

4. Place the glass tissue homogenizer in a rack capable of holding it upright stably.
5. Slowly transfer the contents of the clotted blood tube to the glass homogenizer.

6. Insert the glass pestle and gently homogenize the clotted material and serum. Avoid rapid homogenization strokes as this will lead to excessive foaming and protein denaturation.

7. Remove the pestle to a clean, disposable surface.

8. With a pipettor, transfer a portion of the homogenized clot and serum onto the Blood Card. Be careful to add the liquid to the cutting slowly to avoid drips and runoffs.

9. Transfer unused homogenate back into the original tube and replace the tube closure. Tape the tube closure with laboratory tape. Discard the pipette tip in a biohazard container.

10. Treat the glass homogenizer and pestle with 10% bleach solution. Wash the glass homogenizer with detergent and rinse copiously with water.

11. Permit the bloodstain to dry thoroughly at room temperature.

12. Place the dried stain in a labeled envelope (containing, at a minimum, the Laboratory number and sample identification (K) number) and store at 4EC or -20EC until used.

21.2.2 From Anticoagulated Blood

1. Label an FTA Blood Card with, at a minimum, the Laboratory number and sample identification (K) number.

2. Place a disposable leak-resistant mat beneath the Blood Card.

3. Invert the tube of blood several times to mix the contents.

4. Remove the tube closure and place it on a disposable surface.

5. With a pipettor, transfer a portion of the liquid blood onto the cotton sheeting cutting. Be careful to add the liquid to the cutting slowly to avoid drips and runoffs.

6. Discard the pipette tip in a biohazard container.

7. Permit the bloodstain to dry thoroughly at room temperature.

8. Place the dried stain in a labeled envelope (containing, at a minimum, the Laboratory number and sample identification (K) number) and store at 4EC or -20EC until used.

Rev. #	Issue Date	History
0	06/09/06	Original document issued.
1	11/12/07	Editorial changes made throughout the document. Deleted former Section 2.2.2 Quantification Critical Reagents and renumbered subsequent sections. Deleted water baths as they are no longer used. Allow for purchase of new certified thermometers in Section 2.3.8. Deleted former Section 4 Procedure for Pre-Amplification Quantification, and all subsections and renumbered subsequent sections. Replaced individual dNTP's with pre-mix to simplify PCR set-up. Updated Section 4.3 to reflect that amplification reaction volume may be adjusted prior to CE quantification but not after. Replaced DNA 1000 Series LabChip Kit and DNA 7500 Series LabChip Kit with upgraded Series II LabChip Kits in Section 5. Added safety precautions to include hazards associated with Agilent kits in Section 5.1.1. Replaced instructions for Agilent BioSizing Software with upgraded version of Agilent 2100 Expert Software in Section 5.1.3. Addition of another sequencing primer, H557, for Whole Control Region Sequencing in the presence of HVIII length heteroplasmy in the 568-573 region in Section 6.1. Addition of protocol for half volume sequencing reactions to conserve amplification product when possible in Section 6.1. Replaced instructions and all references for 3100 Genetic Analyzer with upgraded version of 3130xl Genetic Analyzer instructions in Section 7. Updated instructions and references for Sequencher version 4.1.4Fb19 with upgraded version 4.7 in Section 8.1.2. Changed interpretation of comparisons involving non-overlap of HVII length variants in Section 8.3.3. Changed version of mtDNA database from 1.3 to 1.4 due to software upgrade in Section 8.4.2 and subsequent sections. Updated Reagent/Materials/Equipment in Section 10, and suppliers in Section 11.
2	03/26/08	Updated Section 2.4.5 to reflect new NIST SRM number and procedure for sequencing the SRM. Added newly validated procedure for using BigDye Terminator v1.1 Cycle Sequencing Mix to Section 6.1. Added instructions for entering information on sequencing sample sheet when using BigDye Terminator v1.1 Cycle Sequencing

Mix to Section 7.10. Updated Section 9.15 to reflect new policy on not providing sequence results for National Missing Person DNA Database reports. Added BigDye Terminator v1.1 Cycle Sequencing Mix to Section 10.1. Updated information for NIST SRM in Section 19.1. Update of section 21.1 to reflect whole control region sequence for HL60 positive control.

Approval

DNAU II Unit Chief Alice R. Isenberg Date: 3/21/08
Alice R. Isenberg

QA Approval

Quality Manager Robert B. Stacey Date: 3/21/2008
Robert B. Stacey

Issuance

DNAU II QA Program Manager Susan N. Cropp Date: 3/21/08
Susan N. Cropp